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REGULATION OF MACROPHAGE FUNCTION DURING INTRACELLULAR INFECTION WITH *LEISHMANIA DONOVANI*

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ABSTRACT

Investigation of the molecular alterations of macrophage function during intracellular infection by *Leishmania donovani* revealed both adverse and positive influences of this protozoan on host cell function. Chapter I delineates a negative effect which this parasite has on signal transduction pathways in its host cell. In macrophages put in contact, or infected with *L. donovani*, c-*fos* gene expression mediated through protein kinase A was unaffected under conditions where there was an impairment of protein kinase C-mediated c-*fos* gene expression. Selective impairment of protein kinase C-, or, calmodulin-dependent protein kinase-mediated signal transduction in the macrophage was found to influence the establishment of infection. Chapters two and three describe a positive enhancement of macrophage function by *L. donovani*. Intramacrophage infection with *L. donovani* was shown to enhance host cell viability in the absence of growth factor. This was attributable to the elaboration of a soluble factor(s) by infected macrophages into the cell culture supernatant, which enhanced macrophage viability in a manner independent of cell replication. Further characterization of the mechanism of this enhancement revealed that *L. donovani* infection, and lipophosphoglycan, inhibited macrophage death by apoptosis. Cell supernatants derived from *L. donovani* infected cells were also capable of inhibiting macrophage apoptosis. To identify the active factor in infected cell supernatants, the cytokine gene expression profile of *L. donovani* infected macrophages was delineated and possible candidate cytokines were further investigated. Levels of TNF-α capable of causing an abrogation of apoptosis were found to be produced by infected macrophages. However, antibody neutralization of TNF in infected cell cultures could not reverse the inhibition of apoptosis by *L. donovani*, implicating the involvement of multiple factors in the abrogation of apoptosis by *L. donovani*.

ABREGE

L'infection par le parasite intracellulaire *Leishmania donovani* altère certaines fonctions effectrices et accessoires du macrophage. L'étude de ces alterations a démontré que le parasite peut avoir des effets positifs et négatifs sur les fonctions de la cellule hôte. Le chapitre I traite des conséquences négatives de l'infection sur les voies de transmission de signaux internes. Chez les macrophages infectés ou mis en contact avec le parasite, l'expression du proto-oncogène c-*fos*, lorsque médiée par la voie de la proteine kinase A, n'était pas affectée, tandis que celle médiée par la proteine kinase C était altérée. Il a été démontré que l'altération sélective des voies de transmission de signaux internes chez la cellule hôte influence l'établissement de l'infection. Les chapitres II et III décrivent les effects positifs de l'infection sur le macrophage. En effet, l'infection stimulait la viabilité de la population de cellules hôtes en absence de facteur de croissance. Ce phénomène était attribuable à la production d'un facteur soluble par les cellules infectées. Une caractérisation plus poussée du mécanisme a montrée que la mort du macrophage par apoptose était inhibée par *Leishmania donovani*, ou par le lipophosphoglycan du parasite. Il a été demontré que les surnageants de culture des cellules infectées inhibaient également l'apoptose. Afin d'identifier le facteur actif présent dans ces surnageants, le profil d'expression des gènes de cytokines a été élaboré pour les macrophages infectés. Des niveaux de TNF capables d'abroger l'apoptose des macrophages étaient produits par les cellules infectées. Cependant, la neutralisation des molécules de TNF dans le milieu de culture par des anticorps spécifiques n'inversait pas l'inhibition de l'apoptose médiée par le parasite, ce qui implique que plusieurs facteurs seraient attribuables à ce phénomène.

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First and foremost, I would like to thank my research supervisor, Dr. Greg Matlashewski, whose direction and support made this possible. Thank you for your patience, your generosity, and your ever-ready words of encouragement which helped me through the rough spots.

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I would like to dedicate my thesis to my grandfather, Mr. Eric Burnell-Jones, who graduated from Macdonald College in 1938. Sam - I felt you beside me the whole way through.

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STATEMENT OF ORIGINALITY

MANUSCRIPT I:

1. ***Leishmania donovani* selectively impairs PKC-, but not PKA-mediated macrophage signal transduction to the nucleus.** In macrophages put in contact, or, infected with *L. donovani*, c-*fos* gene expression mediated through protein kinase A was unaffected under conditions where there was an impairment of protein kinase C-mediated c-*fos* gene expression. Parasite-cell contact was required for this impairment, as treatment of macrophages with *L. donovani*-conditioned medium was not sufficient to impair PKC-mediated signal transduction.

2. **Impairment of macrophage signal transduction is biologically relevant with respect to infection with *L. donovani*.** Impairment of macrophage PKC-mediated signal transduction enhanced parasite uptake, and resulted in heavier infection levels. Impairment of the calmodulin-dependent protein kinase produced lower parasite burdens upon infection with *L. donovani*, which was likely the result of the selective toxicity of this inhibitor toward the parasite.

MANUSCRIPT II:

3. **Macrophages infected with *L. donovani* exhibit increased viability in the absence of exogenous growth factor.** Enhancement of host cell viability was *Leishmania*-specific, and required sustained infection of the macrophage.

4. **Macrophages infected with *L. donovani* elaborate a soluble factor(s) which enhances macrophage viability.** Infected cell supernatants enhance macrophage viability, but not cell proliferation, and protein synthesis is required for this process.

MANUSCRIPT III:

5. **Infection of macrophages with *L. donovani*, or, treatment of macrophages with lipophosphoglycan, inhibits apoptosis in the macrophage induced by the removal of growth factor.** Abrogation of macrophage apoptosis was also mediated by cell culture supernatants of *L. donovani*-infected macrophages, implicating the elaboration of a soluble factor by infected cells as the mediator of this inhibition.

6. ***L. donovani* infection of macrophages induces the expression of a number of cytokine genes; TNF-α, GM-CSF, TGF-ß and IL-6.** Of the cytokines induced by *L. donovani* infection, TNF-α and GM-CSF were shown to impair macrophage apoptosis.

7. ***L. donovani*-infected macrophages elaborate significant amounts of TNF-α, but no GM-CSF, into the culture supernatant.** However, antibody-mediated neutralization of TNF-α did not restore the ability of infected macrophages to undergo apoptosis, implying the involvement of other factors in *Leishmania*-induced inhibition of macrophage apoptosis.

CONTRIBUTIONS OF OTHERS

All of the experiments contained within, with the exception of the inhibitor studies in manuscript I, were designed and performed by myself under the guidance of Dr. Greg Matlashewski. The inhibitor studies were executed by Ms. Sylvie Labreque, however, I aided in their design and the data analysis.

The research comprising manuscripts II and III was carried out, in part, at the University of Kentucky in the laboratory of Dr. Sam Turco, who provided *L. donovani* promastigotes and lipophosphoglycan.

My supervisor, Dr. Greg Matlashewski, provided advice and feedback on the design and analysis of all three studies, and in the preparation of the manuscripts for publication.

LIST OF FIGURES

MANUSCRIPT III:

Intracellular Infection by *Leishmania donovani* Inhibits Macrophage Apoptosis

LIST OF ABBREVIATIONS

BMM	Bone marrow-derived macrophage
CaM-PK	Calmodulin-dependent protein kinase
CSF	Colony-stimulating factor (GM)= Granulocyte-Macrophage (M)= Macrophage
CT	Cholera toxin
DAG	Diacylglycerol
dBcAMP	Dibutyryl cyclic adenosine monophosphate
IFN-γ	Interferon gamma
IL	Interleukin
IP_3	Inositol triphosphate
LCM	L929 cell-conditioned medium
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
Mϕ	Macrophage
MHC	Major histocompatibility
OAG	1-oleoyl-2-acetyl-glycerol
PGE	Prostaglandin of the E series
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RT-PCR	Reverse transcription polymerase chain reaction
TNF-α	Tumor necrosis factor alpha
TGF-ß	Transforming growth factor beta

INTRODUCTION

Within its mammalian host, the protozoan *Leishmania* survives by intracellular parasitization of the macrophage cell, an integral component of the immune system. As such, in addition to its importance as a human pathogen, *Leishmania* has emerged as an excellent model system for the study of the intricacies of the immune response. Research on leishmaniasis in humans and animals over the past decade has resulted in important insights into the mechanisms involved in lymphocyte activation of macrophages. In particular, study of leishmaniasis has greatly expanded the understanding of the differential expansion of T helper cell subsets, eliciting either a protective or disease exacerbating response which ultimately influences the outcome of disease, and the critical role of effector cytokines in mediating macrophage functions during intracellular parasitic infection.

Resolution of leishmanial infection requires the development of effective cell-mediated immunity culminating in the activation of macrophages to a microbicidal state. However in humans and mice, *L. donovani* causes persistent and potentially fatal visceral infections pointing to a breakdown of macrophage function, and macrophage-T cell interaction in parasite-specific immunity. The main objective of the work presented in this thesis was to investigate molecular alterations within the macrophage initiated during host-parasite interaction. Both positive and negative regulations of macrophage function by *L. donovani* which ultimately influence its intracellular survival were discovered and are described within.

CHAPTER I. LEISHMANIA

I.1 Leishmaniasis: the disease

The protozoan *Leishmania* is the etiological agent for the tropical disease leishmaniasis which currently affects 12 million people worldwide, with an estimated incidence of 1.5 million new cases each year [1]. This disease is prevalent on every continent with the exception of Australia, and is endemic in the tropical regions of America, Africa, the Indian sub-continent, as well as the sub-tropics of south-west Asia and the Mediterranean. As such, leishmaniasis is one of six tropical diseases which has been selected by the World Health Organization for extensive research on diagnosis, treatment, and control. The genus *Leishmania* comprises approximately 20 species which are responsible for a broad spectrum of disease, ranging from self-healing localized lesions to disseminating visceral infections [reviewed in 2]. Thus, leishmaniasis has been divided into three distinct syndromes based on the clinical manifestations of disease. Cutaneous leishmaniasis is the most prevalent form, resulting in ulcers of the skin which may take more than a year to heal. The initial symptoms of mucocutaneous leishmaniasis can be similar, however, this is followed by metastasis from a primary cutaneous lesion to the mucosa, which can result in extensive tissue destruction of the nose and mouth. Visceral leishmaniasis, which causes disseminated infection of the lymph nodes, spleen, liver and bone marrow, represents the most severe form of the disease and is nearly always fatal if left untreated.

Human visceral leishmaniasis, also known as Kala-azar, meaning the black plague,

is caused by *Leishmania donovani*. Visceral leishmaniasis accounts for approximately 500,000 of the new cases of leishmaniasis reported each year [1]. Endemic human visceral leishmaniasis is most frequent in children, with children under the age of 10 accounting for approximately 80% of the reported cases in most areas [3]. Kala-azar causes malaria-type symptoms of high fever, weight loss, anaemia. This is accompanied by a characteristic enlargement of the spleen and liver in infected individuals, resulting in distension of the belly, particularly in children who are the most common sufferers of Kala-azar. Case fatality in this severe systemic disease is estimated to be 5-10% of treated individuals, and 100% of individuals who do not receive treatment. In 1991, large epidemics of visceral leishmaniasis in India and Sudan resulted in approximately 75,000 deaths [1]. While current efforts to control leishmaniasis are focused on vector control by insecticide spraying, and early diagnosis and treatment, the development of a vaccine represents the only long-term hope for controlling this disease. However, the development of an acceptable vaccine remains elusive, and the significant increase in the last decade of the number of leishmaniasis cases is the cause mounting concern of health experts.

The intracellular location of the parasite, and the immunosuppressed state of Kala-azar patients make visceral leishmaniasis a challenge to treat. Pentavalent antimonials have been the standard first-line treatment for Kala-azar for over 40 years [reviewed in 4]. However, primary resistance to antimonials as a result of widespread administration over prolonged periods has begun to limit their usefulness. The cardiac and renal toxicity of antimonials, coupled with the requirement of lengthy treatment periods and the high

cost of the drug, present an urgent need for the development of safer, more effective and affordable drugs for the treatment of Kala-azar. In endemic countries, visceral leishmaniasis is increasingly recognized as an opportunistic infection in AIDS patients [5-8]. As the mounting of an effective cell-mediated immune response is critical for the defense against leishmanial infection, AIDS patients who have T cell defects are highly susceptible to visceral leishmaniasis. In Southwestern Europe it is estimated that 50% of visceral leishmaniasis cases in adults are co-infections of HIV [9]. Following successful treatment of Kala-azar patients, protective immunity generally develops to *L. donovani*, although relapses and possible reinfections are found in both normal and immunosuppressed individuals.

I.2 Life cycle

Members of the genus *Leishmania*, belonging to the family *Trypanosomatidae*, are dimorphic protozoan parasites which oscillate between two distinct host-specific developmental stages. Sandflies of the genera *Phlebotomus* and *Lutzomyia* are the arthropod intermediate host, and are the vector for the transmission of this protozoan. The life cycle of *Leishmania* is illustrated in Figure 1 [10]. Within the midgut of the sandfly, *Leishmania* exists extracellularly as the elongated, motile promastigote. The development of infectivity (metacyclogenesis) is characterized by migration of the promastigotes from the midgut to the proboscis of the sandfly. The infective promastigotes are then injected into the bloodstream of a vertebrate host during feeding.

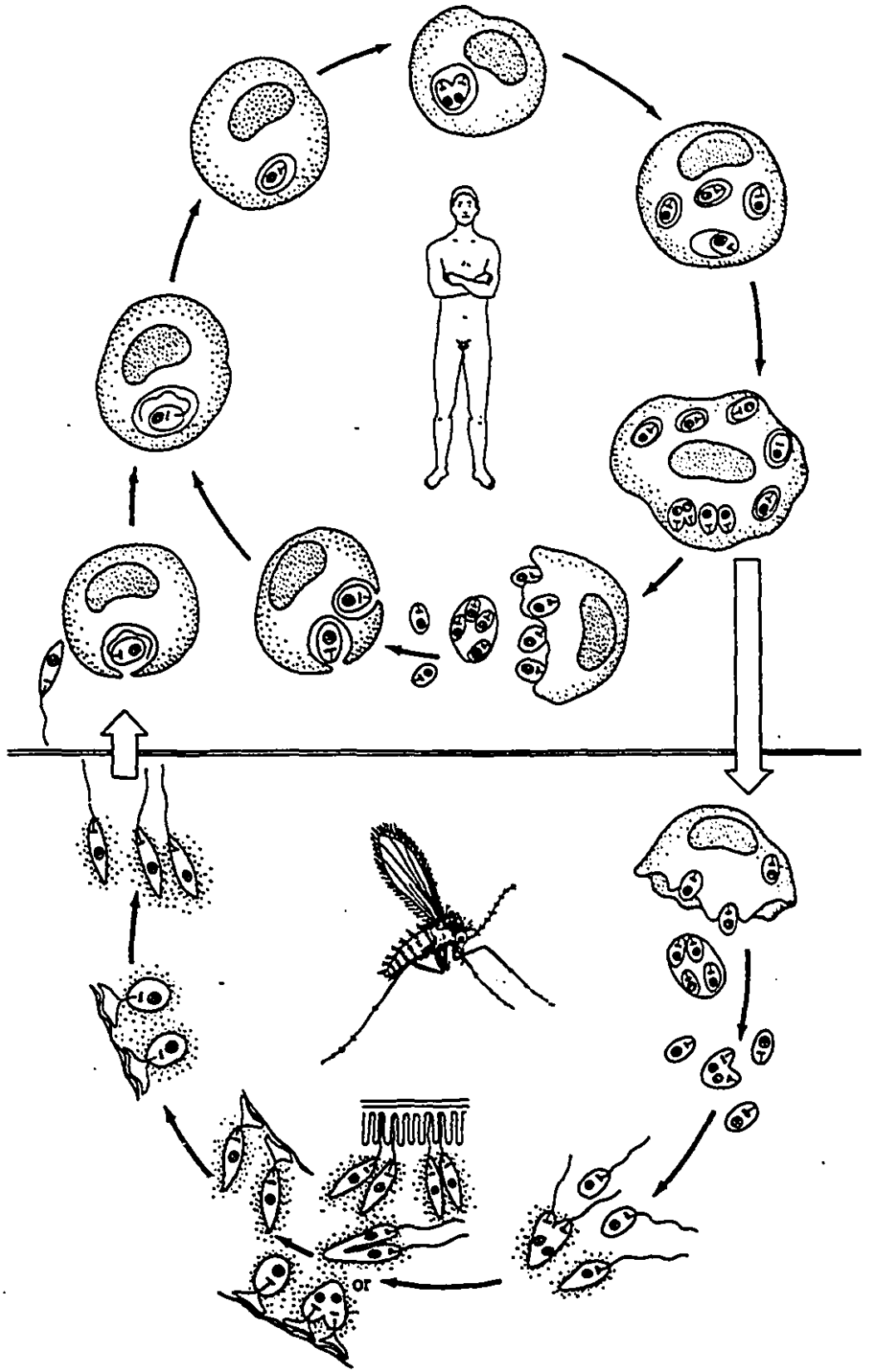

FIGURE 1. Leishmanial infection in sandfly and mammalian hosts

The promastigote form exists very briefly in the bloodstream of the mammalian host, as it is rapidly recognized and ingested by cells of the mononuclear phagocyte system. Within the phagolysosomal compartment of macrophages, the promastigotes rapidly transform into aflagellar, ovoid bodies known as amastigotes. The amastigote form is able to resist destruction in this microbicidal environment, and multiply within the phagolysosome of the macrophage. Intramacrophage replication of the amastigctes eventually results in the rupture of these cells, releasing free amastigotes, which are once again ingested by macrophages. It is this intracellular parasitism of the mononuclear phagocyte system which culminates in the symptoms, and pathology associated with the disease. In visceral leishmaniasis, the parasite initially invades macrophages of the skin, and then subsequently, infection spreads to the lymph nodes, bone marrow, liver and spleen; while in cutaneous leishmaniasis, the parasite remains restricted to the skin and subcutaneous tissue. The life cycle of *Leishmania* is completed when a sandfly takes a bloodmeal from an infected host, taking up parasitized macrophages and free amastigotes from the blood. In the midgut of the sandfly, the amastigotes transform into motile promastigotes and undergo extensive multiplication.

In its mammalian host, *Leishmania* parasites are obligately intracellular. Thus, the survival and transmission of this protozoan is dependent upon the ability to survive within macrophage cells, whose primary role in the immune response is the eradication of invading microorganisms. It is this remarkable ability of *Leishmania* to overcome the macrophage microbicidal mechanisms which make this protozoan such an excellent model for the study of the immunopathogenesis of infectious disease.

I.3 Lipophosphoglycan

Leishmania promastigotes display an abundant glycoconjugate on their cell surface which has been termed lipophosphoglycan, LPG [reviewed in 11]. LPG is localized throughout the surface of the promastigote, including the flagellum [12,13] This novel lipid containing polysaccharide has been implicated in a variety of functions contributing to parasite survival, including providing protection from enzymes in the gut of the sandfly, the lytic effects of complement in the bloodstream, and the hydrolytic enzymes within the phagolysosome of the macrophage.

LPG is a tripartite molecule consisting of repeating phosphorylated oligosaccharide units [PO_4 - 6Gal (ß1,4) Manα1] linked via a glycan core to an unusual glycosylphosphotidylinositol (GPI) anchor [14-17]. In all species of *Leishmania* there is extensive conservation of both the lipid anchor and the phosphosaccharide core region, however, variability exists in the number of repeating saccharide units [18-20]. When grown in culture, structural comparisons between log phase (non-infectious), and stationary phase (infectious) promastigotes have revealed that metacyclogenesis is accompanied by compositional changes of the LPG surface molecules. As the promastigotes differentiate from the logarithmic to the stationary phase of growth, there is an approximate doubling in size of LPG due to an increase in the number of repeating phosphorylated saccharide units [21]. Furthermore, there appears to be an increase in the density of these molecules on the cell surface of the parasite. These structural changes are thought to play significant roles in both the insect vector, and the mammalian host.

In the sandfly, the developmental change in promastigote surface LPG is believed to control attachment and detachment of promastigotes from midgut epithelial cells during development from dividing non-infective forms into resting infective forms, and the subsequent migration to the mouthparts of the insect [22,23]. Examination of epithelial cells from the midgut wall of *Phlebotomus papatasi* revealed that large amounts of non-metacyclic LPG was bound to these cells, however, metacyclic LPG was absent. This suggests that as the LPG matures from its non-metacyclic to its metacyclic form, it loses the ability to bind to the epithelial cells of the midgut, aiding in the migration of infective parasites forward to the mouthparts of the sandfly.

Following inoculation of promastigotes into the bloodstream of a mammalian host, these parasites are vulnerable to the lytic effects of serum. The developmental alterations of LPG during metacyclogenesis appear to represent the major mechanism of resistance to destruction by the host complement system [24]. Non-infectious promastigotes (log phase) are rapidly killed by fresh serum [25], while infectious promastigotes (stationary phase) display an increased resistance to lysis [25,26]. As LPG is the acceptor molecule for C3 attachment on both growth stages of the parasite [26], it has been suggested that the increase in length and abundance of LPG molecules in the metacyclic stage precludes insertion of the complement complex into the promastigote membrane and thus, cell death by lysis [24]. LPG is highly immunogenic, and appears to provide a barrier against the humoral immune response, as Kala-azar serum demonstrates strong reactivity with LPG-deficient mutants of *L. donovani*, but is ineffective against normal promastigotes [27]. In an endemic area in Kenya, it was found that Kala-azar patients cured of infection and

a proportion of the study population with no history of Kala-azar expressed an immune response to purified *Leishmania* LPG [28]. The finding of an immune response in individuals with no history of Kala-azar is consistent with the idea that subclinical infections exist in a proportion of the population.

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CHAPTER II. LEISHMANIA-MACROPHAGE INTERACTION

II.1 Immune Response to Leishmania Invasion

While markedly elevated titers of anti-leishmanial antibodies are a hallmark of Kala-azar [1-4], the humoral immune response appears to have little effect on the resolution of leishmaniasis. Although anti-leishmanial antibodies have been shown to lyse promastigotes in the presence of complement *in vitro*, and to promote phagocytosis, there is little corresponding *in vivo* evidence for any such role for antibodies in the determination of the outcome of infection [5,6]. No correlation was found between antibody titre, or isotype, in the regulation of disease outcome in different strains of inbred mice in experimental leishmanial infection [7,8]. Furthermore, prolonged administration of large amounts of hyperimmune serum, or antibody fractions from mice immunized against *L. major*, failed to influence to outcome of leishmanial infection in highly susceptible BALB/c mice [9]. In addition, depletion of B cells in BALB/c mice resulted in enhanced resistance to *L. major* infection [10].

In contrast to humoral immunity, clinical and experimental evidence for a causal role for the cell-mediated immune response in acquired resistance to leishmaniasis is compelling. Cells of the mononuclear phagocyte system play a dual role in the cell-mediated immune response; macrophages function as antigen presenting cells to mediate the stimulation of T cells, and in turn respond to lymphokines released by activated T cells for microbial killing. The critical role of these interdependent functions in the initiation and regulation of the immune system is clearly illustrated in the case of

intramacrophage parasitism such as leishmaniasis. Resolution of leishmanial infection is dependent on the development of effective cell-mediated immunity, culminating in the activation of macrophages to a microbicidal state. However, *L. donovani* causes persistent and potentially fatal visceral infections in humans, indicating that this protozoan has evolved mechanisms to avoid destruction by the macrophage. The ability of *Leishmania* to survive within a cell noted for its microbicidal properties is shared with several other intracellular parasites, including *Toxoplasma gondii*, *Mycobacterium tuberculosis*, and *Trypanosoma cruzi*. However, unlike *T. gondii* and *M. tuberculosis*, *Leishmania* parasites do not inhibit phagosome-lysosome fusion after ingestion [11,12], nor do they escape from the phagosome as do *T. cruzi* [13,14]. Thus, the amastigote must have adapted to survive, and multiply within this environment which is lethal to most microbial life forms.

Leishmania parasites are initially encountered by the immune system when promastigotes are inoculated into the skin of a mammalian host through the bite of a sandfly. This results in a massive cellular infiltration to the site of entry, where the promastigotes are rapidly ingested by macrophages. In the interim between inoculation and infection of macrophages, promastigotes must avoid destruction by the host's complement system [15]. The major surface molecule, LPG, appears to play a critical role in the resistance of promastigotes to the lytic effects of serum [16,17]. LPG acts as the acceptor molecule for C3 during activation of the alternative pathway of complement, however, its density on the promastigote surface protects *Leishmania* from insertion of complement proteins into the membrane and lysis of the parasite [17-19]. In addition,

L. major promastigotes have now been shown to exhibit externally oriented protein kinases on their surface which phosphorylate C3 and C3b, thereby inhibiting both the classical and alternative complement cascades [20].

II.2 Attachment and Internalization

Attachment and internalization of *Leishmania* parasites has been shown to be a receptor-mediated event [21]. *Leishmania* promastigotes activate complement, and fix C3 on their surface [17,22]. Instead of being detrimental, binding of C3 dramatically increases uptake of *Leishmania* promastigotes, presumably through the complement receptors CR1 and CR3 [22-24]. *Leishmania* uptake through macrophage complement receptors would represent an advantage for the parasite, as CR1- and CR3-mediated phagocytosis occurs in the absence of a respiratory burst [25]. The predominant form of C3 bound to the promastigote surface regulates the use of either CR1 or CR3. Deposition of C3b on the surface of *L. major* metacyclic promastigotes occurs through the classical complement pathway mediating binding to macrophage CR1 receptors [23]. In contrast, *L. donovani* promastigotes bind C3 mainly as inactive C3bi through activation of the alternative pathway of complement, resulting in uptake of the parasite through the macrophage CR3 receptors [24,26-30] LPG and GP63 are the two best characterized molecules on the promastigote surface of *Leishmania* [31]. Both of these molecules have been shown to be acceptor molecules for C3 deposition, and to be involved in promastigote ingestion by macrophages [17,32-35].

In spite of this evidence for a role of C3 in *Leishmania* uptake by complement receptors, several studies have reported serum-independent ingestion of promastigotes via CR3 [26,29,36-38]. In the absence of complement, phagocytosis of LPG-deficient mutants is similar to wild type promastigotes, implying a LPG-, serum-independent pathway for ingestion [39-42]. Ingestion of *Leishmania* promastigotes has been shown to stimulate a respiratory burst in macrophages [43], implying that CR1 and CR3 must not be the exclusive parasite uptake receptors. The mannose-fucose receptor (MFR) of macrophages represents an alternative receptor for parasite uptake, and phagocytosis by this pathway is known to stimulate a respiratory burst [44]. The MFR has been identified in several studies as the receptor involved in *Leishmania* promastigote attachment and ingestion by both murine and human macrophages [26,28,29,34,36,45-47]. Blocking of the MFR of human macrophages inhibited *L. donovani* promastigote attachment by 40%, while blocking of the MFR, in conjunction with the CR3 receptor, inhibited parasite ingestion by 81% [29].

The mechanisms of entry of *Leishmania* reviewed thus far have dealt primarily with the attachment and ingestion of the promastigote stage. By contrast, the receptors involved in amastigote entry of macrophages have not received as in depth attention. Considering the differences in the two stages of *Leishmania*, it is likely that these two distinct forms would have evolved separate mechanisms of entry. Amastigotes of *Leishmania* have been shown to be deficient in LPG [48], or to have altered forms of this molecule on their surface [49,50]. Thus, studies on promastigote attachment can not be directly correlated to amastigotes. Unlike promastigotes, the amastigote form does not

appear to utilize the MFR for macrophage entry [51]. The CR3 complement receptor and the Fc receptor have been implicated as the primary receptors for amastigote attachment and ingestion [51]. In addition to its role in amastigote entry, the involvement of the FcR in the uptake of opsonized promastigotes has been demonstrated [52,53]. However, use of this receptor by promastigotes in the initiation of infection seems unlikely, as parasite-specific antibodies would be absent at this time. In addition, binding to the macrophage FcR would elicit a respiratory burst, to which promastigotes have been shown to be susceptible. By contrast, the FcR could represent one of the major mechanisms of entry of the amastigote into macrophages, as the amastigote form demonstrates considerable resistance to toxic oxygen molecules, and the release of this stage into the bloodstream during established infection would lead to rapid opsonization.

In summary, the attachment and ingestion of *Leishmania* promastigotes and amastigotes by macrophages appears to involve multiple receptors and recognition molecules, and is mediated by both complement-dependent and independent mechanisms.

II.3 Evasion of Macrophage Killing

After internalization of the parasite by the macrophage, the parasitophorous vacuole fuses with the lysosome, and the environment of the phagolysosome becomes acidified [54]. It is thought that the ability of *Leishmania* to survive within the highly toxic phagolysosome is dependent on the capacity of the promastigote to rapidly transform into the highly resistant amastigote [55,56]. While promastigotes of

Leishmania survive optimally at neutral pH, the amastigote form has adapted to regulate physiological activities optimally at pH 4.0-5.5, including respiration, catabolism of energy substrates, and incorporation of precursors into macromolecules [57].

Within the phagolysosome, amastigotes are exposed to toxic oxygen metabolites, amino acid esters, and hydrolases. Amastigotes demonstrate increased resistance to reactive oxygen intermediates generated during the macrophage respiratory burst, compared to promastigotes [58]. This is thought to be due to the greatly upregulated levels of endogenous catalase, peroxidase and superoxide dismustase in the amastigote form [59-61]. In addition, the promastigote molecule, LPG, has been shown to be a potent scavenger of oxygen radicals [62], and appears to play a significant role in the subversion of oxygen-dependent microbicidal mechanisms (discussed in detail in Chapter III.3). Promastigote LPG may also confer resistance to microbicidal lysosomal enzymes [63]. The *Leishmania* surface protease, gp63, has also been shown to degrade host lysosomal enzymes, and displays optimal activity at acid pH [64]. Thus, amastigotes appear to have evolved multiple mechanisms to evade the microbicidal agents within the phagolysosomal compartment of the macrophage, an environment which is highly toxic to most other microorganisms.

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CHAPTER III. IMMUNE RESPONSE TO LEISHMANIASIS

III.1 Genetic Regulation of Leishmaniasis

The understanding of the impact of leishmaniasis on the immunological response of the host has been greatly expanded by the availability of inbred mouse strain models that are susceptible to most *Leishmania* species pathogenic to man. Infection of different strains of inbred mice with *Leishmania* species results in a spectrum of leishmaniasis, as seen in humans. Susceptible murine species develop visceralizing and fatal disseminating disease, while local self-curing cutaneous lesions are induced in resistant strains. In mice, the innate susceptibility to visceral leishmaniasis caused by *L. donovani* is determined by the Lsh gene of chromosome 1 [1]. This gene, which has recently been renamed N*ramp*, controls natural resistance to several intracellular parasites, including *Salmonella typhimurium* and *Mycobacterium* species, and codes for an integral membrane protein [2]. N*ramp* has structural homology with known prokaryotic and eukaryotic transport systems, and a role in transmembrane diffusion of reactive nitrogen intermediates has been proposed for this protein [2]. Increased expression of the *Lsh* gene occurs 2-3 days post-infection with *L. donovani*, both *in vivo* and *in vitro*, and corresponds with an inhibition of parasite multiplication [3,4]. In addition to *Lsh*, at least three additional genes regulate immunity to *L. donovani* in mice; the H-2 linked gene *Rld-1*, an H-II linked gene and *Ir*-2 [5-7]. While *Lsh* gene control influences susceptibility to *L. donovani*, this gene appears to have little involvement in the genetic control of *L. major* infection in mice [8]. Murine infection with *L. major* in susceptible

mice, leads to a progressive course of visceral leishmaniasis which closely resembles human Kala-azar caused by *L. donovani*. Susceptibility to *L. major* infection in mice appears to be controlled by three genes; Scl-1, Scl-2, and the H-11 linked gene which also influences susceptibility to *L. donovani* [6,9]. Use of murine models to study the difference between susceptibility and resistance to leishmaniasis has greatly expanded knowledge on the nature of the cell mediated immune response.

III.2 T Cell Immune Response

The fundamental importance of cell mediated immunity in the resistance to *Leishmania* has long been documented. Early studies in murine models established the critical role of T cells in the cell mediated immune response by demonstrating exacerbation of disease in resistant animals rendered T cell deficient [10-13]. Further experiments established that the ability to control *Leishmania* infection could be restored by reconstitution with $CD4^+$ T cells [14-16], and that adoptive transfer to naive mice of $CD4^+$ T cells from recovered, or immunized mice, resulted in protective immunity [17-20].

In the past decade research on T cell immunology has focused on the segregation of $CD4^+$ T cells into two subsets, Th1 and Th2, on the basis of partially non-overlapping, differential cytokine profiles. Leishmaniasis has greatly aided in defining the relationship between the functional expression of these T helper populations, and disease outcome. In response to stimulation, Th1 clones secrete IL-2, IFN-γ and lymphotoxin, whereas

Th2 clones secrete IL-4, IL-5, IL-6 and IL-10 [21-24]. Both cell types are capable of producing IL-3, GM-CSF and TNF-α. A strong correlation has been demonstrated between susceptibility to murine leishmaniasis and the preferential expansion of the Th2 subset, whereas resistance and resolution of infection is associated with stimulation of a Th1 cell response [25,26].

The dichotomy in the T cell response to *Leishmania* infection has provided a model for the study of the factors that regulate the differentiation of precursors into Th1 and Th2 subsets. Early evidence for T cell heterogeneity in leishmaniasis came from studies of *L. major* infection in highly susceptible BALB/c mice. $CD4^+$ cell depletion at the time of infection reversed the susceptible phenotype of these mice [27-29], and this acquired resistance was found to be reversible by transference of T cells from donors with progressive disease. Further *in vivo* experiments confirmed the role of different T cell subsets in leishmaniasis by demonstrating that transfer of Th1 cells into T cell-deficient mice enabled resolution of infection, whereas transfer of Th2 cells exacerbated the course of disease [25,26].

In murine models of *L. major* infection, there is now clear evidence at both the mRNA and protein levels that $CD4^+$ cells from susceptible strains express high levels of IL-4, but little IFN-γ, whereas $CD4^+$ T cells from resistant strains express high levels of IFN-γ, but no detectable IL-4 [30-32]. Immunological manipulation of the expression of these cytokine at the time of infection has been demonstrated to alter the pattern of the T helper cell response to *Leishmania*. Injection of IFN-γ [32], or neutralizing anti- IL-4 antibodies [33], into susceptible mice at the time of infection with *L. major* drives the T

cell response towards Th1, and resistance to disease. Conversely, administration of anti-IFN-γ antibody to resistant mice render these animals susceptible to infection with *L. major* [34]. To alter the course of disease progression, antibody administration was required close to the time of infection, implicating the alteration of the Th subset response as the mechanism of action, rather than the blocking of the effector function of the lymphokines. Interleukin-4 has been shown to inhibit the macrophage activating potential of IFN-γ *in vitro* [35,36], and local administration of rIL-4 into resistant mice resulted in a transient development of a Th2 response [37]. Together, these studies provide strong evidence for the requirement of IL-4 in the development of a Th2 disease exacerbating response.

Administration of IFN-γ to susceptible mice after the initiation of infection could not modify the progression of disease, suggesting that factors other than IFN-γ are required to influence Th1 cell expansion [32,33]. One such factor appears to be the T cell growth factor, IL-12, which is produced by monocytes and B cells [38]. IL-12 induces IFN-γ production [39-41], and has been shown to play an important role in the initiation of the Th1 response in the resolution of experimental leishmaniasis [42,43]. Treatment of BALB/c mice with rIL-12 during the first week of infection with *L. major* resulted in clearance of the parasite, and this reversal of the susceptible phenotype was attributed to a shift in the T cell response from Th2 to Th1 predominance. IL-12 treatment resulted in a marked depression of IL-4 production, in association with an increase in IFN-γ production. A role for IL-12 in the determination of the outcome ofinfection has been proposed, as treatment of resistant C57BL/6 with anti-IL-12 antibody

resulted in disease exacerbation, and a shift towards a Th2 response [42]. In addition, treatment of macrophages from resistant mice with LPS resulted in the generation of IL-12 levels 40 fold higher than those observed in macrophages of susceptible mice [43]. Together, these experiments imply that IL-12 plays a strong role in the initiation of the Th1 cell response, and the resolution of experimental leishmaniasis. In vaccine trials, IL-12 was demonstrated to be an effective adjuvant for the initiation of protective cell mediated immunity against *L. major* infection by promoting the development of leishmanial specific Th1 cells [44].

Two additional Th2 derived cytokines have been characterized for their ability to influence the T helper cell response in experimental leishmaniasis; IL-10 and IL-6. IL-10 treatment of human macrophages infected with *L. donovani* was found to inhibit cytokine-induced macrophage leishmanicidal activity [45]. IL-10 is a potent macrophage deactivator and has been shown to downregulate Th1 function and IFN-γ secretion [23]. Another Th2 derived cytokine, IL-6, suppressed macrophage killing of *L. amazonensis* in response to IFN-γ and TNFα [46]. The nomination of a role for these cytokines in disease exacerbation is supported by the observation of increased serum levels of both IL-6 and IL-10 in visceral leishmaniasis patients [45,46].

While there is extensive evidence for the role of T helper cell subsets in the determination of the course of murine visceral leishmaniasis caused by *L. major*, a similar regulation of murine leishmaniasis caused by *L. donovani* has been difficult to prove. In a study involving three murine models of *L. donovani* infection, no evidence was found for the overexpansion of Th2 type cells, or for the production of Th2-restricted

cytokines [47]. However, *Leishmania*-reactive T cells with Th1 and Th2 type lymphokine patterns was recently reported in humans who had recovered from visceral leishmaniasis caused by *L. donovani*, representing the first evidence of an analogy with murine models for the existence of dichotomy in T cell response to *Leishmania* [48]. The theory that differential patterns of lymphokine production play a determining role in human regulation of *L. donovani* infection is supported by reports of elevated levels of IL-4 in the plasma of Kala-azar patients [49], and by the promising results of combined IFN-γ/antimonial treatment of visceral leishmaniasis [50].

Production of Inorganic Oxidants

In response to microbial invasion, macrophages produce two independent classes of inorganic oxidants which act as microbicidal defence mechanisms: reactive oxygen intermediates, ROIs [51], namely $O_2^{\cdot}$ and H_2O_2, and reactive nitrogen intermediates, RNIs [52,53], namely NO, $NO_2^{\cdot}$, and $NO_3^{\cdot}$. Both of these classes of inorganic oxidants have been shown to play a role in the killing of *Leishmania.*

III.3 Reactive Oxygen Intermediates

The respiratory burst of macrophages is one of the first lines of defence of this cell to invading pathogens. Phagocytosis perturbs the plasma membrane of the macrophage, resulting in a rapid increase in the rate of oxygen consumption in the NADPH-oxidase reaction, and the production of reactive oxygen intermediates, such as

O_2^- and H_2O_2. These reactive oxygen intermediates are highly toxic, and induce injury through their damaging effect on multiple cellular targets, including membranes, DNA, mitochondria and various cytosolic enzymes. The production of these reactive oxygen products has been correlated with an enhanced ability to kill bacteria, parasites, and tumor cells, and this cytotoxic action has been shown to be enhanced by IFN-γ [54].

For a long time, reactive oxygen intermediates were thought to be the major macrophage killing mechanism in leishmanicidal activity. Attachment and entry of both *Leishmania* promastigotes, and amastigotes, elicit a superoxide generating respiratory burst [55,56]. ROIs have been shown to be toxic for log-phase promastigotes, however, the intracellular amastigote appears to be able to withstand this antimicrobial mechanism [57,58]. The resistance of amastigotes to reactive oxygen intermediates has been postulated to be due to the greatly upregulated levels of endogenous catalase, peroxidase, and superoxide dismutase of this form [59,60]. Indeed, in a macrophage-free system amastigotes removed significantly greater amounts of H_2O_2 than promastigotes [61]. Amastigotes appear to have evolved a further mechanism of evading intracellular killing by ROIs following establishment of infection, by inhibition of the oxidative burst [62]. The major surface molecule of promastigotes, LPG, has also been proposed to play a role in the neutralization of ROIs. LPG is known to bind a subunit of C3, inducing entry of *Leishmania* through the C3 receptor which does not elicit a respiratory burst [63,64]. *In vitro*, LPG alone inhibits the respiratory burst of phorbol myristate acetate-, or zymosan-stimulated macrophages [65,66], and is capable of directly scavenging oxygen radicals [67].

Despite these numerous reports of evidence for the role of reactive oxygen intermediates in the killing of *Leishmania*, it was demonstrated that *L. major* could be eliminated by a murine macrophage cell line (IC-21) deficient in the production of oxygen metabolites [68]. This evidence for the existence of non-oxidative *Leishmania* killing mechanisms is supported by the potent microbicidal activity of liver Kupffer cells to *L. donovani*, despite an inability to generate a respiratory burst [69-71]. Similarly, no correlation was found between leishmanicidal capacity of lymphokine-activated macrophages to *L. enrietti* and *L. major* promastigotes, and the production of ROIs [72,73]. There is now considerable evidence that the oxygen-independent killing mechanism involves the production of reactive nitrogen intermediates.

III.4 Reactive Nitrogen Intermediates:

The production of nitric oxide by activated macrophages has been established as a central effector mechanism against intracellular prokaryotic, and eukaryotic intracellular pathogens [74,75]. Nitric oxide is derived in macrophages from the terminal guanido nitrogen of L-arginine [76,77], through a reaction catalyzed by an inducible nitric oxide synthase [78,79]. Nitric oxide synthase activity can be induced in macrophages by exposure to cytokines, including IFN-γ and bacterial LPS [80-83], and through the synergistic cooperation of IL-2, TNF-α, and LPS with IFN-γ [84-86]. Nitric oxide is very unstable, and rapidly reacts with itself, H_2O, and O_2 to generate a second radical, NO, and finally, the stable end products NO_2^- and NO_3^-. Collectively, these products of

nitric oxide are known as reactive nitrogen intermediates (RNI). The metabolic basis of nitric oxide cytotoxicity appears to be through selective inactivation of vital enzyme systems, as a result of the iron-scavenging properties of this compound [87,88]. Studies involving murine tumor cell lines demonstrated that nitric oxide inhibits three iron-containing mitochondrial enzymes; aconitase of the Krebs cycle, NADPH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase of the electron transport chain [76,89]. Nitric oxide may also inhibit DNA synthesis via inhibition of ribonucleotide reductase [90,91], and this radical may react with superoxide anion to form peroxynitrite, which undergos homolytic fission to yield highly toxic oxidant products [92].

The production of nitric oxide by cytokine-activated macrophages has been correlated with resistance to infection with *Leishmania,* as well as other obligate intracellular pathogens, such as *Toxoplasma*, and *Mycobacterium* [93-96]. Macrophages exposed to IFN-γ produce moderate levels of NO, however, exposure to a second signal such as LPS, or TNF-α or ß, results in a synergistic upregulation of NO production to microbicidal levels [80,81,97,98]. This synergistic influence of IFN-γ, with either LPS or TNF-α, to increase NO production, correlated with the development of potent leishmanicidal activity [23,99]. The direct role of L-arginine-dependent nitrogen oxidation in cytokine-activated macrophage killing of *Leishmania* has been confirmed by several methods. Macrophage cytotoxicity against *Leishmania* is directly dependent upon the availability of extracellular L-arginine *in vitro*, and depletion of L-arginine by arginase inhibited the antileishmanial activity of macrophages [100]. Use of arginine analogues, such as L-N^G-mono-methyl-arginine (L-NMMA), to inhibit the production of

RNIs from L-arginine has been shown to inhibit both leishmanicidal activity, and NO_2^- production *in vitro* [99,101], while injection of this analogue *in vivo* resulted in larger lesion size, and greater parasite loads [100,102]. In addition, the ability of nitric oxide to kill *L. major* promastigotes *in vitro* has also been demonstrated in a macrophage-free system, by treatment with nitric oxide gas, or the NO generator, sodium nitroprusside [100].

As with tumor cells, the proposed targets of NO in antileishmanial activity are enzymes with labile iron prosthetic groups, necessary for DNA replication, the citric acid cycle, and mitochondrial replication. This is supported by evidence that exogenously added $FeSO_4$ inhibits macrophage-mediated antileishmanial activity [101]. *Leishmania* parasites have an additional target enzyme which would be susceptible to NO inactivation, superoxide dismutase. Unlike the SOD of mammals and bacteria, the SOD of *Leishmania* contains an iron group, rendering it susceptible to NO, and inactivation of this enzyme would markedly increase parasite vulnerability to ROIs [103].

Natural resistance/susceptibility to *Leishmania* appears to correlate with the ability of macrophages to generate RNIs in response to infection. Macrophages from resistant strains of mice were found to have higher levels of NOS, and to generate larger amounts of NO when activated *in vitro* [104]. Administration of L-NMMA to genetically resistant C3H/HeN mice inhibited endogenous nitric oxide synthesis, rendering them susceptible to *L. major* infection [102]. Similarly, BMMs from mice of the *Lsh*^{r} genotype demonstrated increased NO production in response to IFN-γ and LPS stimulation, as compared to *Lsh*^{s} mice [105,106].

The regulation of macrophage NOS activity and NO production by cytokines is complex, involving two opposing pathways. Treatment of macrophages with IFN-γ, TNF-α, MIF or IL-7, in conjunction with a co-stimulator, leads to induction of nitric oxide synthase activity. On the opposite side of the avenue, TGF-ß, and the Th2-derived cytokines IL-10 and IL-4, repress nitric oxide synthase activity. The involvement of two opposing pathways allows for tight regulation of the production of nitric oxide.

While the majority of reports have suggested that an additional signal is required in conjunction with IFN-γ for the production of leishmanicidal levels of nitric oxide, several reports have shown that *L. major*-infected macrophages exposed to IFN-γ alone killed the parasite. This effect was correlated with increased levels of NO_2^-, and the absence of the required second signal was thought to be due to the production of endogenous TNF-α in response to *L. major* infection [99,107,108]. TNF-α alone has been shown to activate peritoneal macrophages to kill *L. major* amastigotes *in vitro* [109], and this correlated with an increase in NO_2^- levels [110,111].

Migration inhibitory factor (MIF), which was first characterized for its ability to inhibit the migration of macrophages *in vitro* [112], has now been shown to have antimicrobial and tumoricidal capacities [113,114]. Treatment of human macrophages with rMIF has been shown to activate these cells to kill *L. donovani* [115], and to inhibit the growth of intracellular *Mycobacterium avium* [116], both of which are known to be susceptible to nitric oxide killing. Murine macrophages were activated, in a dose-dependent manner, by rMIF to express nitric oxide synthase, and to produce high levels of nitric oxide *in vitro* [117]. Induction of nitric oxide synthesis by MIF is synergistic

with IFN-γ, but not LPS, and is inhibitable by TGF-ß. In addition to nitric oxide production, treatment of both human and murine macrophages with MIF induced enhanced secretion of TNF-α, which may play a role in MIF-induced killing [115,116].

IL-7 is a potent stimulator of cells of the monocyte lineage capable of inducing macrophage tumoricidal activity and secretion of cytokines, such as IL-6, IL-1α/ß, TNF-α and MIPß [118,119]. Treatment of murine macrophages infected with *L. major* with rIL-7, in the absence of any other stimulus, resulted in parasite killing attributable to nitric oxide production [120]. IL-7 induced killing of *L. major* was synergistically increased in the presence of IFN-γ. Parasite killing induced by IL-7 could be neutralized by subsequent treatment with anti-TNFα antibody, implicating endogenous TNFα production by IL-7 treatment as a central factor in the induction of nitric oxide.

IL-10, a cytokine product of Th2 cells, has received considerable attention as a suppressor of Th1-related IFN-γ production [121,122]. In addition to downregulating the Th1 response, IL-10 is a potent macrophage deactivator capable of inhibiting cytokine-induced nitric oxide production, and nitric oxide synthase activity [123-125]. Incubation of rMIF with IL-10 inhibits MIF-induced macrophage killing of *L. donovani*, and nitric oxide production by human macrophages [125]. Patients acutely infected with *L. donovani* demonstrated IL-10 production which was abolished following successful chemotherapy [126]. IL-10 has been shown to downregulate macrophage microbicidal activity and nitric oxide production against several other intracellular parasites *in vitro* in addition to *Leishmania*, including *T. cruzi* and *T. gondii* [127,128].

The potent macrophage immunosuppressor, transforming growth factor ß (TGF-ß),

is induced in response to infection with *L. braziliensis*, *L. amazonensis*, as well as the intracellular pathogens *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Mycobacterium avium* [129-132]. The production of TGF-ß by *Leishmania*-infected macrophages was found to determine *in vivo* susceptibility to experimental *Leishmania* infection [129]. TGF-ß is known to inhibit the induction of macrophage nitric oxide synthesis by IFN-γ [133-135], and treatment of *L. major*-infected macrophages with TGF-ß was shown to suppress cytokine-activated killing [136], which correlated with an inhibition of nitric oxide production [132].

T helper cell subsets appear to exert an influence at the level of nitric oxide production. Macrophages from a resistant mouse strain, which were pretreated with IL-4 prior to activation and subsequently infected with *L. major*, demonstrated significantly reduced leishmanicidal activity, correlating with an inhibition of NOS activity [137]. Thus, the production of IL-4 by Th2 cells may represent a mechanism by which these cells down-regulate the host-protective nitric oxide response.

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CHAPTER IV. SIGNAL TRANSDUCTION

The activation of cellular functions by external stimuli occurs by transmembrane signalling from the cell surface to the nucleus, through a cascade of biochemical events. The interaction of a ligand with the appropriate cellular receptor initiates signal transduction via a variety of secondary messengers, resulting in specific protein phosphorylation. Phosphorylation of target proteins by kinase enzymes is a basic mechanism for the modification of protein function in eukaryotic cells. Multiple signal transduction pathways have been identified to date, differing in the use of kinase enzymes, and secondary messengers. Two of the main intracellular signalling systems to be implicated in controlling macrophage function are the protein kinase C (PKC) and protein kinase A (PKA) pathways.

IV.1 C-fos Gene Expression

The response of a cell to external stimuli involves the transcriptional induction of several cellular genes. The proto-oncogene, c-*fos*, is among the 'immediate early genes', which are rapidly induced during the stimulation of cells with a wide variety of ligands. Expression of c-*fos* is rapidly and transiently activated in a variety of mammalian cell types, by a diverse set of stimuli, including serum, specific growth factors, phorbol esters, and agents that elevate the secondary messengers calcium and cAMP [reviewed in 1]. In macrophages, c-*fos* expression is differentially inducible via the PKC-, and the

PKA-signal transduction pathways. As with most other cell types, exposure of macrophages to activators of PKC results in a rapid and transient increase in c-*fos* mRNA levels [2]. By contrast, protein kinase A-mediated c-*fos* induction in macrophages is stable for several hours [3]. Studies of c-*fos* upstream regulatory sequences have identified distinct DNA binding sequences for c-*fos* induction. The serum response element (SRE) is the target for at least two distinct signal transduction pathways triggered by growth factors, one of which is protein kinase C-dependent [4-5]. Induction of c-*fos* transcription by agents that elevate intracellular concentrations of cAMP, or calcium, involve the cAMP response element (CRE) sequence within the c-fos promotor [6-7]. Activation of c-*fos* transcription is independent of novel protein synthesis [8], and c-fos expression is greatly amplified in the presence of protein synthesis inhibitors [9], implying that c-*fos* mRNA levels are under post-transcriptional control. In most cell types, c-*fos* induction is transient, due in part to transcriptional retroinhibition by the Fos protein itself [10].

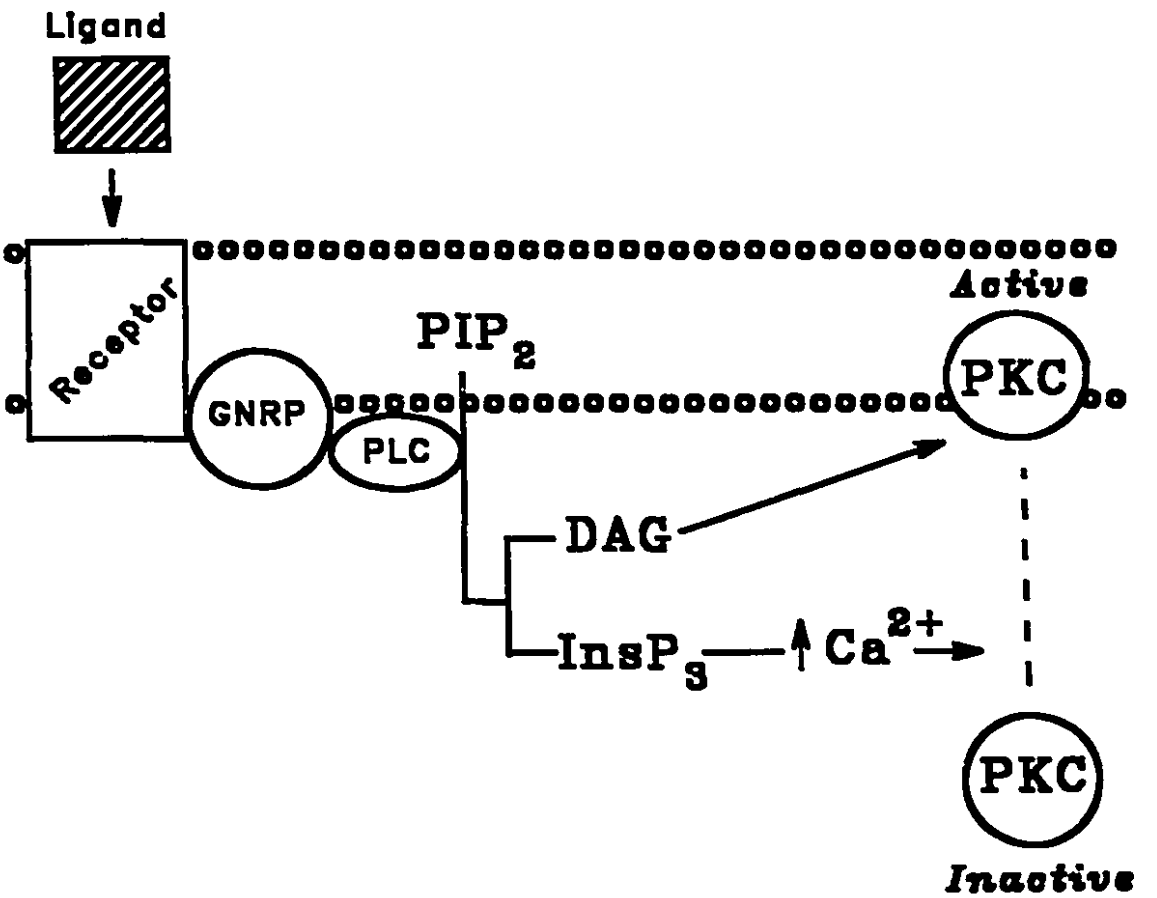
The c-*fos* gene product is a nuclear protein which associates with another proto-oncogene product, c-jun, to form the transcription factor AP-1 [11-12]. AP-1 binding sites have been identified within the promotor regions of a large number of genes [13], and AP-1 transcriptional activity is thought to play a role in cell growth and differentiation [1]. A rapid induction of c-*fos* expression has been shown to occur during differentiation of myeloid cells to macrophages [14], and macrophage activation [15]. The proposed role of c-*fos* in macrophage activation and differentiation, in addition to its differential expression in macrophages, establish this proto-oncogene as a suitable marker for the study of intracellular signal transduction.

IV.2 Protein Kinase C

The PKC family of closely related isozymes mediates ATP-dependent phosphorylation of a wide range of target proteins on specific serine and threonine residues [16,17]. This kinase is primarily cytosolic, and activation results in translocation of the enzyme to the membrane, in a calcium-dependent fashion [18,19]. The PKC signal transduction pathway is illustrated in Figure 2. The PKC pathway is initiated by ligand binding of specific cellular receptors, triggering phospholipase C (PLC) activity via a G protein-coupled mechanism [20-22]. Activated PLC catalyzes the hydrolysis of membrane phosphatidylinositol bisphosphate (PIP_2), generating the secondary messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with intracellular membranes releasing calcium into the cytosol, while DAG is the physiologic activator of cytoplasmic PKC, inducing its translocation to the membrane and subsequent protein phosphorylation. The appearance of DAG is fleeting, lasting only seconds, or at the most minutes, after its formation [17]. Thus, PKC is active for only a brief time after receptor interaction. However, the consequence of PKC activation, ie. protein phosphorylation, may persist for a long time, depending on the biological stability of the phosphate attachment. DAG greatly increases the affinity of PKC for calcium, thus activation can occur without significant increases in intracellular calcium concentrations [23]. This observation is significant, as IFN-γ was recently reported to induce a slow rise in DAG as a result of phospholipase D action on phosphotidylcholine [24]. This would represent an alternative pathway for PKC activation independent of PLC-phosphoinositide metabolism.

Figure 2:

Protein Kinase C Signal Transduction



Protein Phosphorylation

- Oxidative burst
- MHC Class Ia expression
- c-fos gene expression
- IL-1 production

Potential roles for PKC in the control and modulation of many metabolic processes have been proposed. Proteins activated by phosphorylation through PKC include DNA topoisomerase II [25], and the transcription factors AP-1 and $NF_{\kappa}ß$ [26], which may in turn induce many additional genes. In addition, PKC has been shown to modulate several important macrophage activation-associated functions, including expression of MHC class II Ia expression [27], IL-1 production [28], and induction of the respiratory burst [29,30].

IV.3 Protein Kinase C and Leishmania

For an intramacrophage parasite such as *Leishmania*, targeting of the PKC signal transduction pathway would represent a powerful tool to subvert macrophage activation and subsequent killing. Indeed, *Leishmania*-infected macrophages have been shown to exhibit numerous defective PKC-associated responses *in vitro* [31-33]. Macrophages infected with *L. donovani* demonstrated markedly attenuated protein phosphorylation after PMA-induced stimulation of PKC [33]. This defect in stimulus-response coupling in infected macrophages was attributed to altered kinase activation by DAG and PKC translocation. This effect could be partially attributable to the promastigote cell-surface glycoconjugate, LPG, since LPG could inhibit PKC activity in live macrophages [34]. In addition, *Leishmania* LPG has been shown to be a potent inhibitor of PKC *in vitro* [35,36]. Purified LPG of *L. donovani* was found to selectively impair enzyme activity of PKC, but not PKM, or PKA, isolated from rat brain. This specific inhibition of PKC,

but not PKM, which represents the catalytic domain of PKC, implies that LPG interacts with the regulatory domain. This domain contains the binding sites for DAG, calcium and phospholipids. Correspondingly, LPG was found to be a competitive inhibitor of DAG, and a non-competitive inhibitor of phosphatidylserine [35]. Treatment of macrophages with LPG *in vitro*, attenuated OAG-induced PKC phosphorylation of two target proteins examined [34]. Further studies examining the effect of LPG on PKC translocation revealed that LPG treatment of macrophages had no effect on the association of this enzyme with the membrane [34]. However, since *L. donovani* amastigotes have little, or no LPG, it remains unclear how the PKC enzyme is inhibited in *L. donovani*-infected macrophages.

Consistent with reports that *Leishmania* parasites can inhibit PKC, is the observation that infected macrophages exhibit impaired oxidative burst upon stimulation [33,37,38]. Impairment of the PKC-mediated respiratory burst may play a significant role in the establishment of infection by promastigotes, as this form of the parasite has been shown to be susceptible to toxic oxygen metabolites [39-41]. Indeed, depletion of PKC in macrophages by prolonged PMA treatment [16], rendered these cells susceptible to infection with *L. donovani* promastigotes *in vitro*, and inhibited lymphokine-activated killing of amastigotes in established infections [42,43]. LPG has been proposed to play a significant role in *Leishmania*-mediated impairment of PKC-mediated respiratory burst for enhanced promastigote survival [44]. Pretreatment of cells with LPG, or phagocytosis of LPG-coated beads, resulted in attenuation of the respiratory burst in PMA stimulated monocytes, as measured by oxygen consumption [44,45].

Two additional PKC-dependent macrophage responses important for accessory cell activation have been shown to be suppressed in *Leishmania*-infected macrophages; IL-1 production and MHC Class II expression. These two independent macrophage functions are pivotal in the induction of anti-leishmanial T cell activation [46]. Elaboration of IL-1 by macrophages in response to microbial challenge is a principal mediator of the host immune response. This activation-associated function was found to be suppressed during *Leishmania* infection [47]. Attachment and penetration of *L. donovani* promastigotes to macrophages *in vitro*, failed to induce IL-1 synthesis. In addition, *Leishmania*-infected macrophages demonstrated impaired capacity to respond to stimuli for IL-1 production [47,48]. In response to LPS, macrophages infected with either *L. major* or *L. donovani* demonstrated significantly reduced levels of IL-1 production compared to uninfected cells. Gene expression studies revealed that while *L. donovani* infection did not induce IL-1ß mRNA accumulation in macrophages, the induction of IL-1ß mRNA by LPS stimulation was not impaired in infected macrophages [49]. These results implicate a post-transcriptional mechanism for the attenuation of LPS-induced IL-1 production in *Leishmania*-infected macrophages. Thus, these findings indicate that *Leishmania* has evolved mechanisms to subvert macrophage IL-1 production upon initial infection, and to suppress the ability of infected macrophages to produce this cytokine in response to stimuli. Unresponsiveness to LPS with respect to IL-1 production, has also been demonstrated in macrophages pretreated with LPG [45]. Thus, this cell surface glycoconjugate of *Leishmania* promastigotes may play a significant role in the observed attenuation of LPS-induced IL-1 production in *Leishmania*-infected macrophages.

Macrophage surface expression of MHC Class II Ia, in conjunction with foreign antigen, represents a critical function for T cell recognition. Macrophages infected with *L. donovani* or *L. major* were found to demonstrate impaired antigen presenting capacity *in vitro* [50-53]. Investigation of antigen presentation by *L. major*-infected macrophages revealed that inhibition of antigen presentation could not be attributed to impaired antigen processing, but indicated that the presence of *L. major* may interfere with the intracellular loading of MHC Class II molecules with antigenic peptides [50]. Macrophages infected with *L. donovani* were unresponsive to IFN-γ stimulation for the induction of MHC Class II antigen expression [51,42], and gene expression analysis revealed that IFN-γ induced MHC Class II Ia α and ß mRNA was markedly reduced in infected macrophages [52,53]. Thus, *L. donovani* infection suppressed parasite-specific antigen presentation by MHC Class II by downregulation of lymphokine induction of MHC Class II mRNA. As expression of MHC Class II antigen has been proposed to be required for IL-1 production [46], the inhibition of MHC Class II by *Leishmania* may explain the concurrent abrogation of IL-1 production.

Further examination of PKC-dependent signal transduction in *L. donovani*-infected macrophages revealed impaired expression of c-*fos* gene expression [31]. In response to LPS, *L. donovani*-infected macrophages exhibited an approximate 50% reduction in the level of mRNA,as compared to uninfected macrophages. Direct stimulation of PKC using OAG did not abrogate this impairment. In addition, impairment of LPS-induced TNF-α gene expression was observed in *L. donovani*-infected macrophages [31], however, it remains unclear whether PKC is involved in this signal transduction pathway

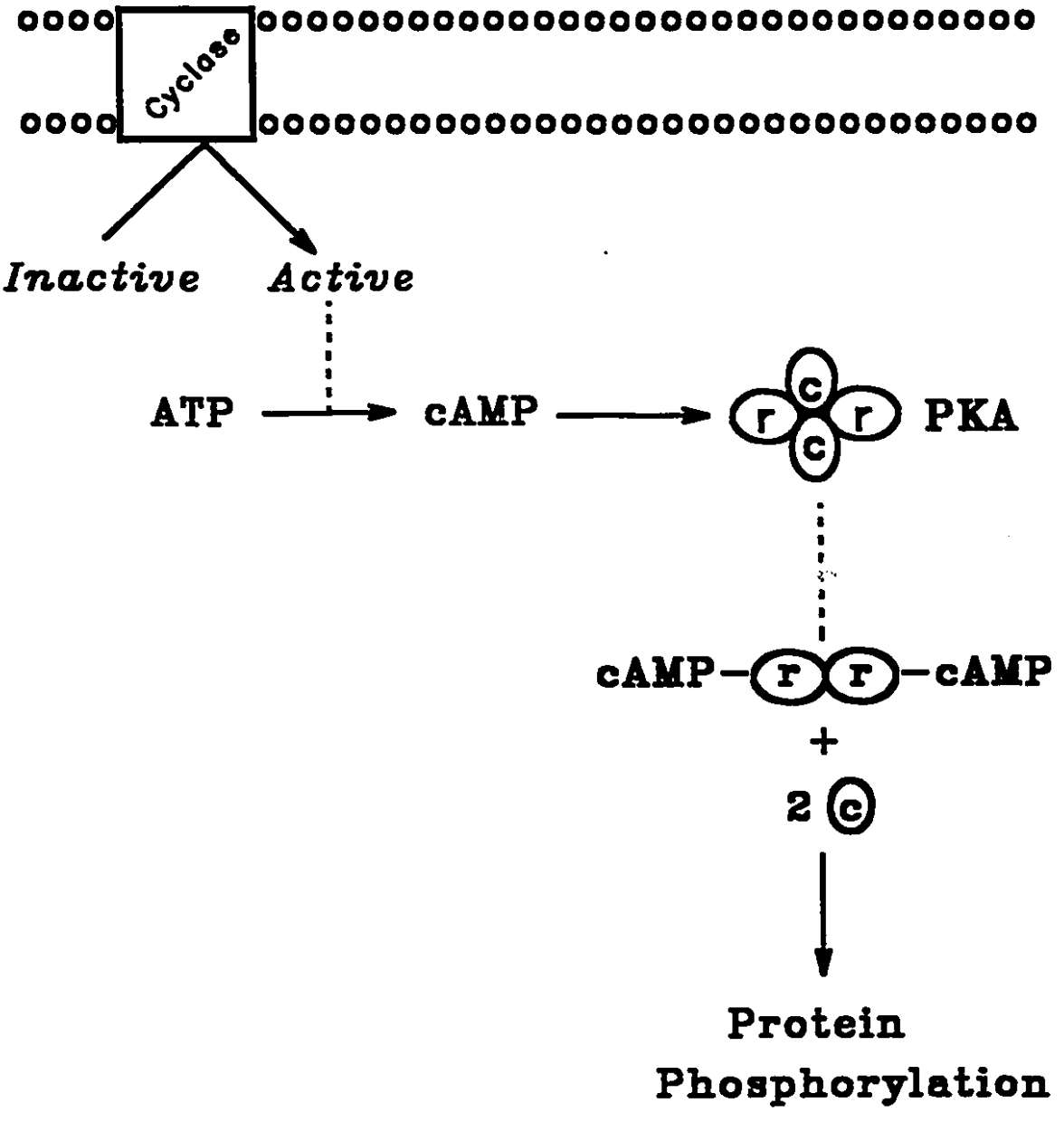
[28]. Impairment of TNFα gene expression in infected macrophages was found to be reversible by treatment with indomethacin, implying that this impairment was mediated by increased levels of cyclooxygenase products by *Leishmania* infection [31]. In further studies, it was shown that pretreatment of BMMs with LPG alone resulted in inhibition of LPS-, and OAG-induced c-fos gene expression in a dose-dependent manner [55]. This effect of LPG was selective, as no effect on LPS-induced TNF gene expression was observed in LPG-treated macrophages.

IV.4 Protein Kinase A

The secondary messenger cAMP mediates cellular metabolism by activation of the protein kinase A signal transduction pathway, resulting in protein phosphorylation and regulation of gene transcription [reviewed in 56,57]. The cAMP-PKA pathway is illustrated in Figure 3. PKA signal transduction is initiated by ligand binding of an adenylate cyclase-coupled receptor. Receptor-ligand complexes activate cyclase activity via unique regulatory G proteins, which transduce both stimulatory and inhibitory signals [58]. Induction of adenylate cyclase activity catalyzes the conversion of ATP to cAMP, which in turn activates PKA, resulting in phosphorylation of protein substrates on specific serine and threonine residues. Protein kinase A is a holoenzyme which exists as an enzymatically inactive tetramer, composed of two catalytic and two regulatory subunits. When levels of intracellular cAMP are elevated, cAMP binds to the regulatory subunits, and the holoenzyme dissociates, yielding a regulatory subunit dimer and two active

Figure 3:

Protein Kinase A Signal Transduction



catalytic subunits. The hormonal activation of adenylate cyclase can be pharmacologically mimicked by the administration of stable analogues of cAMP, such as dibutiryl cAMP or 8-bromo-cAMP. Stimulation of adenylate cyclase activity through guanine nucleotide-binding proteins can be mediated by cholera toxin [59,60], or directly, by the diterpene forskolin [61].

Activation of the catalytic subunit of PKA results in specific protein phosphorylation, and induction of cAMP-regulated gene expression [62,63]. Transcriptional regulation by PKA occurs by phosphorylation of the cAMP response element-binding protein (CREB), which can initiate transcription of other genes containing cAMP response elements (CRE) within their promoter/enhancer regions [64-66]. Agents that increase intracellular cAMP levels lead to an induction in c-*fos* mRNA [67-69]. In most cell types, induction of the c-*fos* gene results in transient expression. In macrophages, induction of c-*fos* gene expression by cAMP results in stable transcription for several hours [3]. The presence of the CRE in the c-*fos* promoter-enhancer region is responsible for the induction of c-*fos* gene expression by cAMP [6].

cAMP was first identified over 30 years ago as an important secondary messenger regulating cellular responses to external stimuli. Since its discovery, extensive studies on the function of the cAMP-PKA signal transduction pathway have demonstrated that PKA activation downregulates many aspects of the immune response, leading to the general consensus that this pathway mediates an "off signal" to effector cells [56]. Prostaglandins of the E series have been shown to be potent inhibitors of the immune response, and their action has been attributed to stimulation of the adenylate cyclase

complex and accumulation of intracellular cAMP [70]. PGE_2 has long been known to have an inhibitory effect on T cell proliferation and differentiation regulated by IL-2 [71-74]. In addition, triggering of the PKA pathway has been shown to inhibit a number of key macrophage functions, including phagocytosis [75], oxygen radical production [76-78], IL-1 production [79], TNF production [80], MHC Class II Ia expression [81,82], tumoricidal activity [83], leukotriene B_4 synthesis [84], and plasminogen activator secretion [85]. Cyclic AMP analogues, as well as cAMP inducing agents such as PGE_2, have been shown to reverse the bactericidal ability of macrophages against *Legionella pneumophila*, *Mycobacterium intracellulare*, and to inhibit proliferation of peripheral blood monocytes in response to *Staphylococcus epidermidis* [86-88].

Stimulation of the PKA pathway has been shown to downregulate expression of several macrophage genes. Treatment of macrophages with cholera toxin, or dBcAMP, strongly suppressed LPS-stimulated expression of the early response genes, JE and TNF [89,90]. In addition, administration of dBcAMP to human promyelocytic cells (HL-60) inactivated transcription of both the c-*myc* and the transferrin receptor genes [91]. In IL-2 treated T cells, treatment with 8-bromo-cAMP inhibited c-*myc* gene expression, as well as the IL-2 receptor and c-*fos* protein synthesis [92]. In addition to this primary PKA-mediated negative influence on gene expression, several studies have indicated that elevated intracellular cAMP levels have a negative effect on receptor-induced PKC activation [93-99]. The mechanism behind this inhibitory influence appears to involve interference at the level of PI breakdown. Agents increasing intracellular cAMP concentrations have been shown to inhibit the formation of both DAG and IP_3, in several

cellular systems [93,94,98,99]. Phosphorylation of PLC-γ by protein kinase A has been proposed to be the origin of this inhibition [100]. Together, these studies depict a role for the involvement of the PKA pathway in both primary and secondary inhibitions of signal transduction in the macrophage activation.

IV.5 Protein Kinase A and Leishmania

Several studies have attempted to establish a role for the cAMP-PKA pathway in the deregulation of cell mediated immunity in response to *Leishmania* infection. Macrophages infected with *L. donovani* were found to produce increased amounts of arachidonic acid metabolites, specifically PGE_2 [54]. As PGE_2 has the potential to inhibit T cell activation, this would represent a potential mechanism for *Leishmania* to inhibit the cellular immune response of the host to infection. Stimulation of the PKA pathway was found to have differential effects on induction of macrophage killing of *L. enriettii* [101]. Addition of PGE_2, or dBcAMP, inhibited calcium ionophore (A23187) induced leishmanicidal activity, but not IFN-γ-mediated killing, in the presence of LPS [101].

PGE_2 is known to inhibit the expression of MHC Class II molecules, presumably through activation of the cAMP dependent PKA pathway [82]. *L. donovani* infection has been shown to stimulate synthesis of PGE_2 by macrophages, representing a potential parasite-induced mechanism for the inhibition of MHC Class II expression [51,54]. Suppression of PGE_2 secretion, using cyclooxygenase inhibitors, partially restored responsiveness of infected macrophages to lymphokine-induced MHC Class II expression

[51]. However, further investigations revealed that despite increased PGE_2 production, concentrations of intracellular cAMP were not altered in *L. donovani*-infected macrophages, and the inhibition of MHC Class II gene transcription by *L. donovani* was demonstrated to be cAMP-independent [53].

To date, information on PKA signal transduction during leishmanial infection is limited, and further studies are required to define the role of prostaglandins and PKA in the immune response to *Leishmania* infection.

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CHAPTER V. CYTOKINES & HAEMATOPOIESIS

V.1. Haematopoiesis

The bone marrow, which is the source of white cells, red cells, lymphocytes and megakaryocytes, is a constantly self-renewing system. The turnover rate of peripheral mature cells is high, and new cells must be continually fed into the system. This intricately balanced process for the production of the different classes of mature blood cells is termed haematopoiesis. Haemopoietic cells of all lineages are derived from a small population of pluripotent stem cells residing in the bone marrow. Regulation of the growth and differentiation of stem cells is mediated by glycoproteins growth factors of the colony-stimulating factor (CSF) and interleukin families (IL) [reviewed in 1].

One characteristic that all of the CSFs share is their ability to maintain the viability of their respective target cells *in vitro* [2], and it was this property for which they were initially described [3]. The CSFs were classified by the mature cell types which they induced and named accordingly; macrophage-CSF, granulocyte/macrophage-CSF and granulocyte-CSF. M-CSF and G-CSF are specific growth factors affecting macrophages and granulocytes respectively, while GM-CSF and IL-3 influence several lineages. The delineation of distinct roles for these cytokines is often clouded by overlapping biological activities and target cells, and highly synergistic interactions [reviewed in 4]. In addition to their action on macrophage survival and differentiation, M-CSF and GM-CSF stimulate the release of many immunological mediators from these cells, including interferons, interleukin-1, prostaglandins and tumor necrosis factor [4].

In the absence of growth factors, haemopoietic cells undergo cell death by apoptosis [5], and suppression of cell death has been proposed as one of the primary functions of the CSFs. The effects of the CSFs on cell survival can be uncoupled from cell proliferation, since exposure to low concentrations of M-CSF maintains macrophage survival, while higher concentrations promote both survival and proliferation [6,7].

V.2 Macrophage-CSF

M-CSF is produced by monocytes, fibroblasts and endothelial cells, and stimulates the formation of macrophages exclusively [reviewed in 8]. M-CSF exists as a homodimer of approximately 70-90 kD, and exerts its influence by binding to a specific macrophage surface receptor, CSF-1R. This receptor exhibits tyrosine kinase activity, and has been shown to be the c-fms proto-oncogene product [9-10]. Activation of the receptor kinase triggers a cascade of events, which in primitive mononuclear phagocytes, leads to entry of the cell into the S phase and cell division [7].

In addition to its role in the proliferation and differentiation of mononuclear phagocytes, M-CSF has multiple effects on mature cells. M-CSF induces the release of multiple inflammatory agents, including plasminogen activating factor, prostaglandin E, interleukin 1, interferon, and tumor necrosis factor [11-14]. In addition, M-CSF can stimulate macrophage tumoricidal activity [15-16], enhance secretion of antimicrobial reactive oxygen intermediates [17], and activate macrophages to kill invading pathogens [18]. Examination of the effect of M-CSF on macrophage killing of *Leishmania* have

yielded conflicting results. While application of rM-CSF was demonstrated to activate intramacrophage killing of *L. mexicana amazonensis* [19], a similar study with *L. donovani* showed no effect of M-CSF on parasite killing [20].

V.3. Granulocyte/Macrophage-CSF

GM-CSF is a 23 kd glycoprotein produced at sites of inflammation primarily by macrophages, fibroblasts, and activated T cells [21]. In addition to its role in maintaining the survival and differentiation of granulocytes and macrophages, this cytokine also regulates diverse functional activities of mature macrophages, including adherence, migration, cytokine expression, oxidative metabolism, and microbicidal and tumoricidal activity [reviewed in 21]. The biological activity of GM-CSF is mediated through the binding to a transmembrane receptor, resulting in internalization of GM-CSF. The GM-CSF receptor consists of an α-chain that binds GM-CSF with low affinity, which can associate with a high affinity ß-chain to initiate signalling through the ligand-receptor complex [22]. In human cells, a common ß-subunit combines competitively with the α-chains of GM-CSF, IL-3 or IL-5 [22,23].

In response to microbial invasion, macrophages release GM-CSF, and this cytokine has been shown to activate macrophages to resist viral infections and to kill intracellular parasites such as *Trypanosoma cruzi* and *Mycobacterium* spp [24-25]. Release of GM-CSF at local sites of infection would result in the recruitment of both macrophages and granulocytes to assist in host defence. The role of GM-CSF in the

outcome of leishmaniasis is controversial. The ability of rGM-CSF to activate macrophages to kill *Leishmania* has been repeatedly demonstrated [26-28]. However, despite this evidence, further studies have demonstrated exacerbation of disease following administration of rGM-CSF [29], contradicting the proposed host protective effect of this cytokine. In addition, higher production of GM-CSF in mouse strains susceptible to *L. mexicana amazonensis* compared to resistant strains has been reported, strengthening the argument that this cytokine may exacerbate disease [30]. GM-CSF was determined to be a growth factor for *L. mexicana amazonensis*, providing promastigote protection from heat induced death [31-32].

V.4. Tumor Necrosis Factor α

The pleiotropic cytokine, TNF-α, is a primary mediator of inflammation, produced predominantly by macrophages [33]. Paradoxically, macrophages are also one of the main targets of action for TNF. TNF-α downregulates M-CSF cell surface receptors [34], stimulates macrophage cytokine production [35,36], acts as an autocrine intermediate in macrophage microbicidal action [37,38], and interacts synergistically with other cytokines to stimulate macrophage proliferation [39,40]. The effects of TNF are mediated through the activation of two distinct cell surface receptors of the same non-tyrosine kinase receptor family; a 55-60 kd, and a 75-80 kd receptor species [41,42]. These two TNF receptors possess markedly different intracellular regions, suggesting disparate signalling pathways and functions.

TNF has been demonstrated to play an important role in mediating host protection against cutaneous leishmaniasis. Treatment with rTNF *in vitro* activated macrophages to kill intracellular *L. major*, and *in vivo* administration of rTNF induced resolution of infection [43-45]. Earlier reports also attributed a leishmanicidal role to TNF, however, a second signal, provided by IFN-γ was required for parasite killing [46]. Treatment of genetically resistant mice with anti-TNF antibody led to partial inhibition of the resistant state, implying that the ability to produce TNF influenced susceptibility to leishmaniasis [43,44]. Despite this evidence, TNF was not detected in cell supernatants of macrophages infected with *L. major* [43,44] or human monocytes infected with *L. donovani* [47]. However, infection of macrophages with *L. major* [44], but not *L. donovani* [47], greatly enhanced the ability of these cells to produce TNF upon stimulation with LPS, and immunization of susceptible mice with avirulent *L. major* resulted in the production of high levels of TNF in response to challenge with a virulent form [44].

Further investigation revealed that TNF provided an essential parasite-induced autocrine signal in the macrophage microbicidal response to *Leishmania*. Treatment of macrophages infected with *L. major* with anti-TNF antibody prevented IFN-γ-induced macrophage activation [37]. This inhibition of leishmanicidal activity correlated with an abrogation of macrophage nitric oxide production, implying that parasite-induced TNF provides an essential signal for the induction of leishmanicidal killing by nitric oxide [37,48,49].

In addition to leishmaniasis, TNF has been shown to be an essential component

of host resistance to numerous other diseases, including viral, bacterial and parasitic infections. TNF mediates anti-viral effects, in many cell lines, against such animal viruses as vesicular stomatitis virus, encephalocarditis virus, adenovirus type 2 and herpes simplex virus 2 [50-51]. Host protective effects mediated by TNF have also been demonstrated in bacterial infections with *Listeria* [52,53], *Salmonella* [54], and *Mycobacterium* [55,56]; in fungal infections with *Torulopsis* and *Candida* [57]; and in protozoan infections with *Plasmodium* and *Trypanosoma* species [58,59]. Conversely, this cytokine has also been shown to have detrimental influences on disease progression in some systems. TNF secretion has been associated with the fatal outcome of cerebral malaria, and the fatal symptoms of bacterial meningitis [60-62].

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CHAPTER VI. APOPTOSIS

VI.1 Apoptosis

Apoptosis is a widespread and morphologically distinct "suicide mechanism" which preserves homeostasis by maintaining the balance between cell proliferation and cell death [1]. Apoptosis, and its suppression, play a fundamental role in both embryonic development and in the maintenance of the integrity of adult tissues. This active process of cellular self-destruction requires specific gene transcription and protein synthesis in the targeted cell prior to death [2-4]. The signal to die appears to come from the environment due to exposure to, or removal of, a specific growth factor or hormone [3-10]. In haemopoietic stem cells, the suppression of apoptosis is primarily mediated by the CSFs [10], which ultimately influence the pathway of differentiation of these cells. Human monocytes undergo apoptosis within 8 h following removal of stimuli [6]. The role of CSFs in the suppression of apoptosis can be uncoupled from proliferation, as exposure to low concentrations of M-CSF promotes survival of macrophages, while higher concentrations promote survival and also stimulate proliferation [11,12].

While the mechanism of activation of apoptosis varies widely with respect to cell type and external signals, once triggered, the process is remarkably similar between tissues and species [13]. This active cell death is morphologically distinct from the passive and unregulated death by necrosis, which ultimately culminates in cell lysis. Cells undergoing apoptosis demonstrate cell shrinkage and extensive nuclear condensation, followed by membrane blebbing, and cell fragmentation [14]. One

hallmark of apoptotic cell death is the fragmentation of genomic DNA into oligonucleosomal-sized fragments. This is mediated by the activation of an endogenous endonuclease which randomly cleaves DNA within the internucleosomal linker region, generating a ladder of DNA fragments of 200bp multiples [15].

In vertebrates, apoptosis is instrumental in regulating the correct functioning of the immune system. Stimulation of the immune system characteristically involves the rapid proliferation of macrophages and lymphocytes. Following immune stimulation, leukocyte homeostasis must be re-established, and it is primarily through selective apoptosis that this equilibrium is maintained. Thus, the central lymphoid organs, such as the thymus and the bone marrow, are the sites of extensive apoptosis [16]. Swift recognition and disposal of dying leukocytes is crucial, as the release of their highly toxic intracellular constituents would result in inflammation and subsequent damage to surrounding tissues. As such, a key feature of programmed cell death is the rapid recognition and phagocytosis of apoptotic cells by macrophages before cell lysis occurs, thereby avoiding leakage of the cell contents [17].

Several individual genes have been implicated in the process of apoptosis. Of these, the proto-oncogenes *bcl*-2 and c-*myc* represent two of the most prominently studied. Overexpression of *bcl*-2 has been commonly detected in B-cell lymphomas, suggesting that this oncogene has 'anti-apoptotic properties' [18-21]. *Bcl*-2 expression has been shown to play a fundamental role in the selection of mature T cells for survival after activation [22]. Transfection of *bcl*-2 into an IL-3 dependent myelopoietic cell line rendered these cells growth factor independent, and induced cell differentiation [23].

Thus, expression of *bcl*-2 appears to protect cell from apoptotic death. The role of c-*myc* in the modulation of apoptosis is more complex. C-*myc* appears to play a role in regulating the choice between proliferation and apoptosis. It has been demonstrated that under conditions unsuitable for proliferation c-*myc* expression induces apoptosis [24-27], but in combination with other stimuli, such as *bcl*-2 expression, c-*myc* expression can lead to transformation [18,27-28]. *In vivo* administration of c-*myc* antisense oligonucleotides have been shown to inhibit activation-induced cell death of T cell hybridomas [29], confirming that c-*myc* expression is likely to be necessary for progression of apoptosis.

The tumor suppressor gene, p53, has also been associated with apoptotic cell death. The p53 gene product arrests cell proliferation following genotoxic assault, presumably allowing time for DNA repair in damaged cells. A crucial role has been demonstrated for p53 gene expression in the execution of thymocyte apoptosis, both *in vivo* and *in vitro* [30-32]. p53 was shown to be an essential component of the pathway from the induction of DNA damage leading to apoptosis [32]. Apoptosis induced by exposure of thymocytes to ionizing radiation did not alter p53 gene transcripts, however, a dramatic increase in protein levels was observed prior to degradation of genomic DNA, implicating a posttranslational control mechanism [30]. Introduction of this tumor suppressor gene into myeloid cell lines normally lacking p53 resulted in rapid loss of cell viability due to apoptosis [33]. This correlates with the role of p53 inactivation in tumour development, as loss of p53 would inhibit non-viable cells from undergoing cell death by apoptosis.

In summary, manipulation of expression of several genes appears to regulate apoptosis. Inhibition of apoptosis is mediated through *bcl*-2 activation or p53 inactivation, while c-*myc* expression induces apoptosis under specific circumstances.

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RATIONALE FOR STUDY

Manuscript I:

Previous studies from our laboratory have demonstrated that infection of macrophages with *L. donovani* inhibited PKC-mediated c-*fos* gene expression, and this was proposed to be mediated in part by the promastigote molecule, LPG [1-2]. It was of interest to determine the effect of *L. donovani* on other signal transduction pathways. The implication of the PKA signal transduction pathway in the downregulation of several aspects of the immune response [3], combined with the finding of increased prostaglandin E_2 secretion by *L. donovani*-infected macrophages [4], made this pathway an intriguing target for study.

While previous studies from our laboratory have also indicated that LPG impaired PKC-mediated c-*fos* gene expression, but had no effect on PKA-mediated c-*fos* gene expression [2], evidence for the absence of this molecule on *L. donovani* amastigotes [5] prompted further study of the effect of live parasites on the PKA signal transduction pathway. Furthermore, the biological implications of the inhibition of signal transduction in the macrophage, with respect to leishmanial infection, remained to be determined. Thus, it was proposed to examine the effect of both contact, and infection, by *L. donovani* promastigotes and amastigotes, on macrophage c-*fos* gene expression differentially induced via the PKC- and PKA-signal transduction pathways. In addition, the effect which various signal transduction inhibitors had on the establishment and maintenance of infection by *L. donovani* was investigated to determine the relevance of impaired signal transduction to infection levels. This work was published in the Journal of Immunology in May 1993 (vol. 150: 4457-4465).

Manuscript II:

Previous studies on the molecular interaction of *Leishmania* and the macrophage have focused primarily on the negative effects which this protozoan has on its host cell. In order to fully characterize the molecular basis of leishmanial infection, both positive and negative influences of infection on the host needed to be investigated.

Throughout our earlier studies, we observed that *L. donovani* appeared to have a positive influence on macrophage viability. Although we began our experiments with similar numbers of cells, throughout our studies we observed consistently more *L. donovani*-infected macrophages, than non-infected macrophages. This represented a potential novel discovery of a positive influence, worthy of further investigation, which this protozoan infection had on its host cell. Thus, it was proposed to characterize the effect of *L. donovani* infection on macrophage viability *in vitro*, and to investigate the mechanism of this influence. This work was published in the Journal of Leukocyte Biology in January 1994 (vol. 55: 91-98).

Manuscript III:

Investigation of the positive influence described in manuscript II, which *L. donovani* infection had on its macrophage host cell, revealed that this protozoan enhanced macrophage viability in the absence of growth factor in a manner independent of cell proliferation. This effect was found to be partially attributable to the elaboration of a soluble factor(s) by *L. donovani*-infected macrophages. This motivated our investigation of alternative mechanisms of enhancing macrophage viability, in particular impairment of apoptosis, and potential macrophage cytokines which could act in an autocrine manner to enhance macrophage viability. This work has been accepted for publication in the Journal of Immunology and will appear in the March 15, 1994 edition.

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SECTION B

MANUSCRIPTS I, II, III

MANUSCRIPT I

ALTERATION OF *LEISHMANIA DONOVANI* INFECTION LEVELS BY SELECTIVE IMPAIRMENT OF MACROPHAGE SIGNAL TRANSDUCTION[1]

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FOOTNOTES:

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2. Abbreviations:

PKC, Protein kinase C; PKA, Protein kinase A; LPG, lipophosphoglycan; Dbcamp, dibutyryl CAMP; CT, Cholera toxin; OAG, 1-oleoyl-2-acetyl-glycerol; BMM, bone marrow-derived macrophage; CaM-PK, Calmodulin dependent protein kinase.

ABSTRACT:

Leishmania donovani is an obligate intracellular protozoan which resides and multiplies in macrophages. The molecular basis for this host-parasite interaction is poorly understood. Targeting a signal transduction pathway in the macrophage would allow this parasite to manipulate cellular gene expression, and this may aid in ensuring its survival. We demonstrate that in macrophages infected with *L. donovani* for 18 h, c-*fos* gene expression mediated through protein kinase A was unaffected under conditions where there was an impairment of protein kinase C (PKC)[2] mediated c-*fos* gene expression. This selective impairment of PKC mediated c-*fos* gene expression was substantially augmented in macrophages put in contact with *L. donovani* promastigotes or amastigotes for only 1 h. Treatment of macrophages with *L. donovani*-conditioned media was not sufficient to impair signal transduction. These data revealed that *L. donovani* selectively impaired the transmission of information from the cell surface to the nucleus and that this effect is induced very soon after macrophage-parasite contact. The biological significance of this altered signal transduction in the macrophage with respect to infection with *L. donovani* was then examined by treating macrophages with various protein kinase inhibitors prior to infection with amastigotes. Macrophages that were treated with PKC inhibitors demonstrated an increase in the initial uptake of the parasite and carried heavier infection levels than did controls. In contrast, treatment of macrophages with an inhibitor of calmodulin-dependent protein kinase (CaM-PK) did not show significant differences in the initial uptake of parasite, but prolonged impairment

of CaM-PK resulted in a decrease in the level of macrophage infection. Further experiments revealed that promastigote proliferation was severely impaired by the CaM-PK inhibitor, but not any of the other inhibitors.

INTRODUCTION:

Leishmania donovani is an obligate intracellular protozoan which colonizes the macrophage system of its vertebrate host, causing visceral leishmaniasis in humans. This parasite is digenetic, existing as flagellated extracellular promastigotes within the gut of its sandfly vector, and as non-motile amastigotes within mononuclear phagocytes of its mammalian host. Resolution of the disease is believed to be the consequence of the cell mediated immune response [1]. Given the role of macrophages in both the initiation and resolution of infection, the survival of this parasite within these cells appears to be assisted by factors that inhibit or reduce the impact of the macrophage microbicidal mechanisms. For example, *L. donovani*-infected macrophages are impaired in their ability to produce IL-1β or express class I or II MHC gene products, both of which are required for the induction of the T cell dependent immune response [2-5]. Infected macrophages also demonstrate an impaired oxidative burst, which is a primary defense of the cell during *L. donovani* invasion [6].

To become activated to resist or kill an infectious agent, macrophages must be capable of responding to external signals by conveying information from the cell surface to the nucleus. Therefore, signal transduction pathways represent an attractive target for an intracellular pathogen, as this would allow the parasite to alter gene expression and thus, the normal activity of the cell without entering the nucleus. The c-*fos* gene represents a useful nuclear marker to examine signal transduction in *L. donovani*-infected macrophages [7-8]. The c-*fos* proto-oncogene is among the immediate early genes which

are expressed immediately following macrophage activation with LPS [9]. Transcription of the c-*fos* gene is also rapidly induced by other mitogens and growth factors, and its product acts as a transcriptional trans-activator by complexing with c-*jun* to interact with AP-1 enhancer sites [10]. Two of the better characterized signalling pathways in the macrophage for c-*fos* gene expression are the PKC- and the PKA-associated signal transduction pathways. PKC-mediated c-*fos* expression in macrophages results in a transient increase in c-*fos* mRNA peaking 30 min following stimulation [11], while PKA mediated c-*fos* gene expression results in stable transcription for several hours [12]. This difference in kinetics of c-*fos* transcription allows gene expression from these two signalling pathways to be easily distinguished. These properties render c-*fos* a suitable reporter gene to examine the effect of *L. donovani* on signal transduction from the cell surface to the nucleus in the macrophage. Recent investigations from this lab have demonstrated that *L. donovani* and its major surface molecule, LPG, were capable of impairing PKC-mediated c-*fos* gene expression [7-8], most likely by impairing PKC activity [13]. LPG did not impair PKA-associated gene expression [8]. It is not known whether promastigotes or amastigotes of *L. donovani* are capable of impairing other signal transduction pathways including that involving PKA, and this is a relevant question since it has recently been shown that the amastigote form of *L. donovani* does not have detectable LPG on its surface [14]. More importantly, the biological implications for impaired signal transduction in macrophage defense against *L. donovani* remain to be determined. We have therefore undertaken to compare the effect of *L. donovani* on c-*fos* gene expression mediated through PKC and PKA and to examine the biological

significance of altered signal transduction in the macrophage with respect to infection with this protozoan.

MATERIALS AND METHODS:

Leishmania donovani

Amastigotes of the Ethiopian LV9 strain of *L. donovani* were recovered from the spleen of infected female Syrian hamsters (Charles River Canada, St. Constant, Que.) as previously described [15]. Promastigotes were derived through amastigote culture in supplemented DMEM at 26°C and were used in the stationary phase of growth.

Cell preparation

Bone marrow cells from 6- to 8-wk-old female BALB/c mice (Charles River Canada) were cultured at 37°C in RPMI complete medium (Gibco/BRL Life Technologies, Burlington, ON) supplemented with 10 mM HEPES, 100 U/mL penicillin and streptomycin, 10% heat-inactivated low endotoxin FCS and containing 15% v/v L929 cell-conditioned medium as a source of CSF-1. After 1 day in culture, nonadherent cells were transferred into new culture dishes and allowed to differentiate for 7 days in L929 cell-conditioned medium. Macrophages were collected and washed three times in HBSS (Gibco/BRL Life Technologies) and resuspended in CSF-1-free medium for 18 h to ensure quiescence before use.

***L. donovani* infection of macrophages**

Macrophages were infected in suspension for 3 to 6 h at an amastigote-to-cell ratio of 20:1. For experiments involving infected macrophages, amastigotes were used instead

of promastigotes, as amastigotes are at least 10 times more infective *in vitro* than are promastigotes. Noningested amastigotes were removed by centrifugation by three washes in RPMI complete medium, and the infected cells were incubated at 37°C for 18 h before use. Infection levels were routinely about 4 amastigotes/cell as evaluated by cytospin and Giemsa staining, with over 90% of the cells infected.

To examine signal transduction after the early interaction of *L. donovani* with macrophages, a modification of the above procedure was used. Macrophages in suspension were exposed to promastigotes or amastigotes at a parasite-to-cell ratio of 10:1 for 1 h. The promastigotes used were non-infective because they had been passaged in tissue culture media for greater than 1 yr. Promastigotes/amastigotes were then removed by centrifugation, the macrophages were washed three times in RPMI complete medium, and then subjected to gene expression analysis. Cytospin samples taken at this time routinely showed that the majority of macrophages remained uninfected (less than 1 amastigote/cell) when treated with amastigotes and contained no infection when treated with promastigotes. No free protozoan were present after the washing.

Treatment of macrophages with *L. donovani* supernatants was performed by placing quiescent macrophages in six-well culture plates overnight. Millipore 0.45 uM culture plate inserts were used as a membrane barrier in treated wells, allowing free diffusion of ions and macromolecules. Promastigotes or amastigotes were added in the culture plate inserts at a parasite-to-cell ratio of 10:1 for 2 h, after which time the inserts were removed and cells were used.

Gene expression analysis

L. donovani treated and nontreated cells were stimulated with the PKA activators, dBcAMP at 1mM for 1 h, and CT at 10 ng/mL for 1.5 h (ICN Biomedicals Canada Ltd., Mississauga, ON), or the PKC activators, 1-oleoyl-2-acetoyl-glycerol (OAG) at 100 uM for 20 min and LPS at 100ng/ml for 30 min (Sigma Chemicals Co., St. Louis, MO). After stimulation, total RNA was extracted using RNAzol B (Tel-Test Inc., Friendswood, TX) which is a modification of the guanidinium-phenol-chlcroform method [15]. Ten ug of total RNA was treated with glyoxal, electrophoresed in 1% agarose and blotted onto Hybond-N membranes (Amersham Canada Limited, Oakville, ON). Blots were hybridized at 42°C in 50% formamide with ^{32}P-labelled nick-translated probes and washed once in 0.5 x SSC (1 x SSC is 0.15M NaCl and 0.015M Na citrate) at room temperature and twice at 55°C. Autoradiography was performed at -70°C with an intensifying screen, using Kodak XAR-5 film (Picker International Canada Inc., St. Laurent, Que.). The c-*fos* probe consisted of the 1.3 kb *Pvu*II-*Bgl*II fragment from pFBH-I [16], kindly provided by Dr. N. Teich, ICRF, London, UK. Blots were reprobed with actin (1.25 kb *Pst*-I fragment of pBA-1) [17], as an internal control for the quantity of total mRNA in each lane. Densitometry analysis was performed to allow for a quantitative comparison.

Inhibitors and infection levels

Macrophages were treated with 20 uM of either H7 or W7 (Seikagaku America Inc., Rockville, MD) for 15 min prior to infection or 100 ng/ml PMA (Sigma Chemicals) overnight prior to infection with *L. donovani* amastigotes for 3 h. Noningested

amastigotes were removed by three washes in RPMI complete medium, and the inhibitors were added back and maintained throughout the course of the study. Cytospin preparations were made at 3, 24, 48 and 72 h after infection to monitor infection levels. The effect of the above mentioned protein kinase inhibitors on promastigote proliferation was monitored for 9 days.

RESULTS:

PKC- & PKA-mediated c-*fos* gene expression in *L. donovani*-infected macrophages

Previous studies from this laboratory revealed that macrophages infected with the protozoan *L. donovani* demonstrated impaired PKC-mediated c-*fos* gene expression [7]. To examine the specificity of this impairment of signal transduction by *L. donovani*, we compared PKA- and PKC-mediated c-*fos* gene expression in infected cells under similar conditions. LPS stimulates PKC-dependent signal transduction to induce a rapid and transient increase in the level of c-*fos* mRNA, which reaches its peak at 30 min [18]. A similar increase in transcription of the c-*fos* gene is induced by the synthetic diacylglycerol OAG, which stimulates the PKC enzyme directly [11]. Conversely, cholera toxin, which exerts its effect on the G-protein/adenylate cyclase complex of the PKA-dependent signal transduction pathway, and dBcAMP which stimulates the PKA enzyme directly, result in stable transcription of the c-*fos* gene for several hours in macrophages [12]. Therefore, infected and control macrophages were treated with LPS and OAG to stimulate PKC-mediated c-*fos* gene expression or treated with dBcAMP and CT to stimulate PKA-mediated c-*fos* gene expression. As revealed in Figure 1A, c-*fos* gene expression mediated through PKA (lanes 7-10) was unaffected in *L. donovani* infected macrophages at a level of 4 amastigotes per macrophage, although the same infected cell population showed an impairment of PKC-mediated c-*fos* gene expression (lanes 3-6). Densitometry analysis (Fig. 1B) of the Northern blot film for c-*fos* mRNA relative to actin mRNA revealed that the impairment of PKC-mediated c-*fos* gene

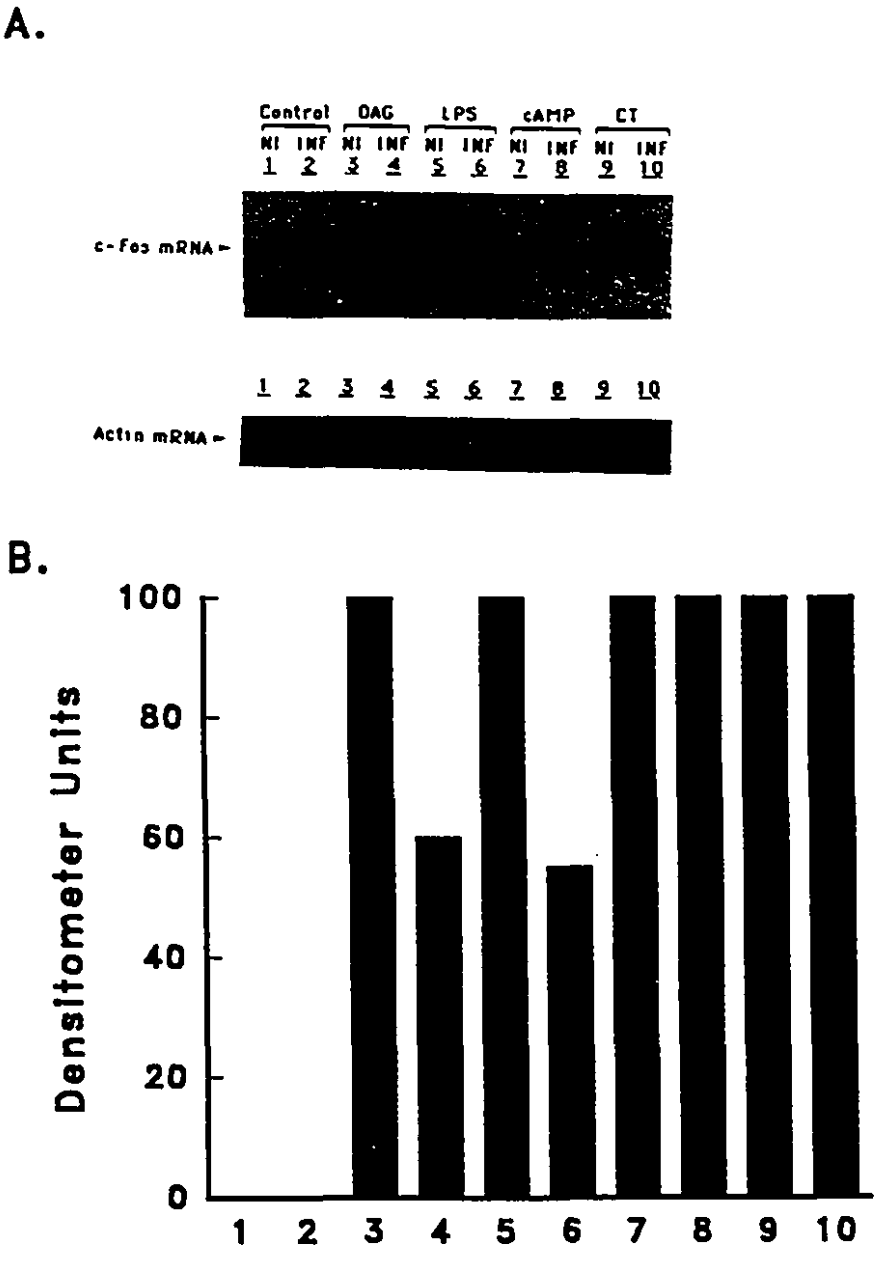

Fig. 1. Effect of *L. donovani* infection on PKC- and PKA-mediated c-*fos* gene expression. Noninfected (odd lanes) and macrophages infected at 4 amastigotes per cell (even lanes) were treated with the PKC stimulators OAG 100 uM (lanes 3,4) and LPS 100 ng/ml (lanes 5,6) or the PKA stimulators dBcAMP 1 mM (lanes 7,8) and CT 10 ng/ml (lanes 9,10) as described in Materials and Methods. Control cells (lanes 1,2) received no stimulation. (A) Total RNA was extracted and c-*fos* and actin mRNA levels were determined by Northern blot analysis. (B) Densitometry analysis of c-*fos* mRNA levels relative to actin mRNA levels. These are representative of three separate experiments.

expression in response to OAG and LPS was 40% and 45%, respectively, and this is consistent with our previous observations [7]. This selective impairment of PKC-mediated c-*fos* expression under conditions where PKA-mediated c-*fos* gene expression was unaffected in infected cells was highly reproducible.

To determine the effect of *L. donovani* on signal transduction during the initial interaction between the macrophage and the parasite when there is a low level or no infection, we examined PKC- and PKA-mediated c-*fos* gene expression in macrophages exposed to the protozoan for only 1 h. At this time, virtually all macrophages remained uninfected as described in Materials and Methods. As shown in Figure 2, macrophages put in contact with either promastigotes or amastigotes for 1 h showed a selective impairment of PKC-mediated c-*fos* gene expression, with little effect on PKA-mediated c-*fos* expression. Under these conditions, there was an almost total impairment of PKC-mediated gene expression by both amastigotes (Fig. 2A, lane 4) and promastigotes (Fig. 2A, lane 8). Densitometry analysis of this Northern blot for c-*fos* mRNA levels relative to actin mRNA revealed that the impairment of PKC-mediated c-*fos* gene expression by amastigotes was 90% and 100% by promastigotes, with little measurable effect on PKA-mediated c-*fos* gene expression (Fig. 2B). This demonstrated that early contact with *L. donovani* promastigotes or amastigotes was more efficient at impairing PKC-mediated gene expression than was prolonged intracellular infection, and in each case, there was a selective impairment of the PKC-mediated signal transduction pathway.

It was necessary to determine whether the results shown in Figure 2 were because of parasite contact with macrophages, or because of a molecule(s) present in the parasite-

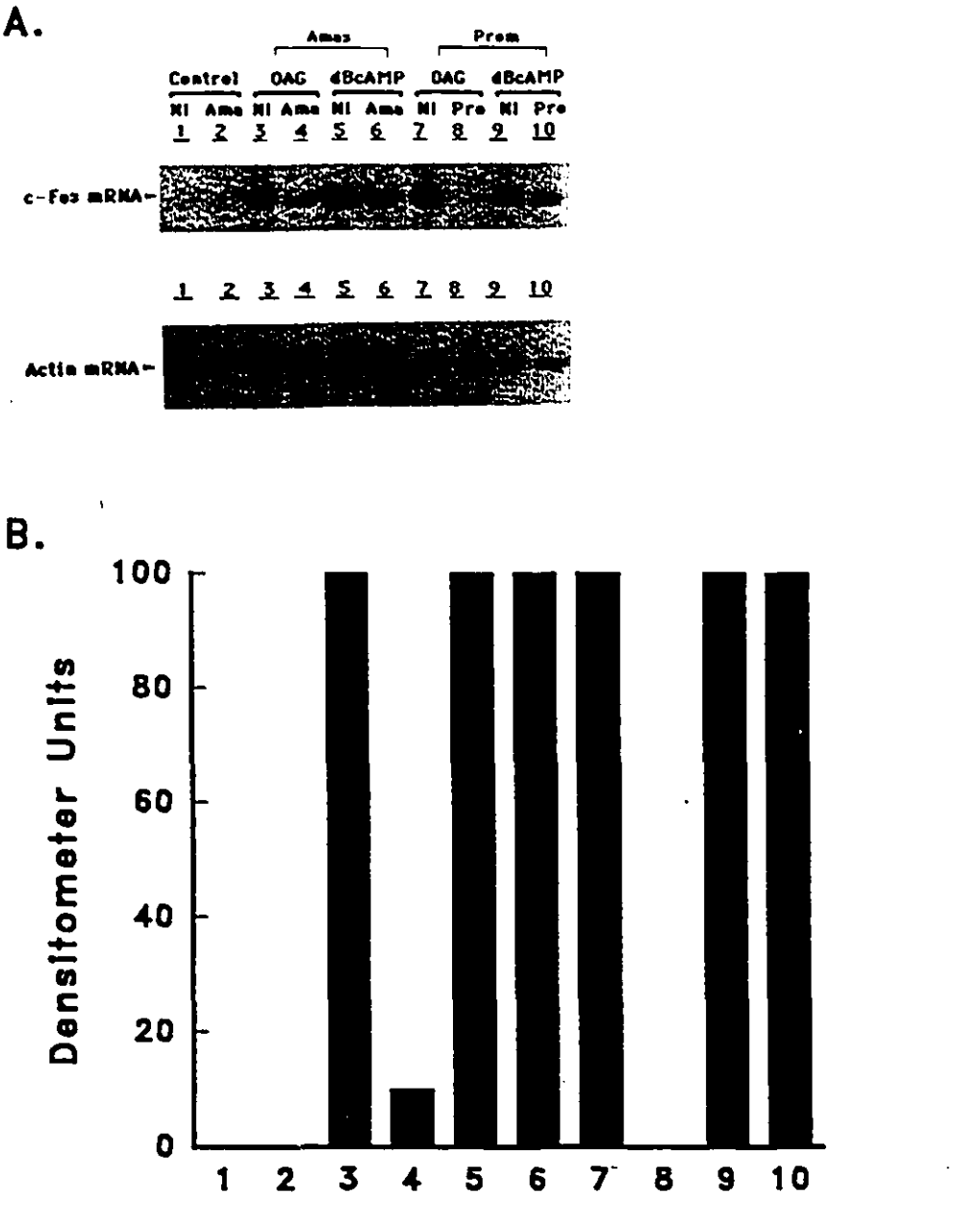

Fig. 2. Effect of *L. donovani*-macrophage contact on PKC- and PKA-mediated c-*fos* gene expression. Normal macrophages (odd lanes) and macrophages treated with amastigotes (lanes 2,4,6) or promastigotes (lanes 8,10) for 1 h were stimulated with the PKC activator OAG 100 uM (lanes 3,4,7,8) or the PKA activator dBcAMP 1 mM (lanes 5,6,9,10) as described in Materials and Methods. Control cells (lanes 1,2) received no stimulation. (A) Total RNA was extracted and c-*fos* and actin mRNA levels were determined by Northern blot analysis. (B) Densitometry analysis of c-*fos* mRNA levels relative to actin mRNA levels. These are representative of three separate experiments.

containing media. Therefore, macrophages were treated with *L. donovani*-conditioned media for 2 h prior to stimulating PKC-mediated c-*fos* expression with OAG. As shown in Figure 3, treatment of macrophages with *L. donovani* amastigote-conditioned media had no effect on PKC-mediated c-*fos* gene expression. Treatment of macrophages with promastigote-conditioned media showed a 15% impairment of PKC-mediated c-*fos* gene (Fig. 3A) expression as determined by densitometry analysis (Fig. 3B). These data argue that parasite-macrophage contact was essential for *L. donovani* to maximally impair PKC-mediated signal transduction.

L. donovani infection in the presence of signal transduction inhibitors

In light of the above data, it was necessary to determine whether the selective impairment of signal transduction was biologically relevant to parasite entry or subsequent survival. It has previously been shown that pretreatment of peritoneal macrophages with PMA markedly impaired the ability of these cells to kill *L. donovani* after activation with lymphokines [20]. More recently, it has been shown that PMA treatment of nonactivated BMM also caused higher infection levels with *L. donovani* [13]. It was, however, of interest to compare impairment of PKC with PMA (a specific inhibitor of PKC) to a more general signal transduction inhibitor to determine whether impairing other kinases will further increase the level of infection. Therefore, to investigate impairment of signal transduction on *L. donovani* infectivity, macrophages were treated with PKC, PKA, and CaM-PK antagonists prior to infection with amastigotes, and the number of amastigotes per macrophage were subsequently monitored over 3 days. The following signal

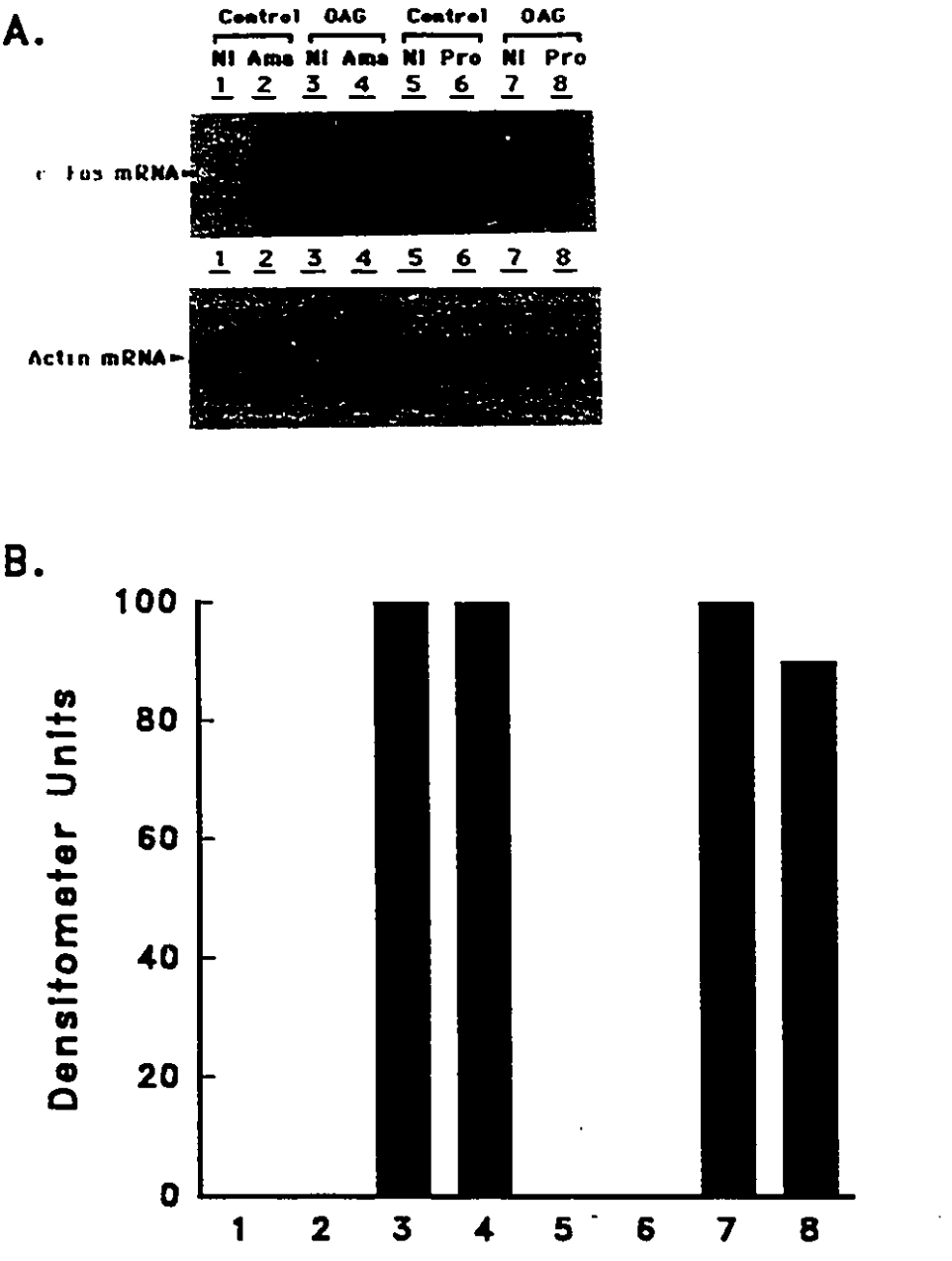

Fig. 3. Effect of *L. donovani*-conditioned media on PKC-mediated c-*fos* gene expression. Normal macrophages (odd lanes), macrophages treated with amastigote-conditioned media (lanes 2,4), or promastigote-conditioned media (lanes 6,8) for 2 h were stimulated with the PKC activator OAG 100 uM (lanes 3,4,7,8) as described in Materials and Methods. (A) Total RNA was extracted and c-*fos* and actin mRNA levels were determined by Northern blot analysis. (B) Densitometry analysis of c-*fos* mRNA levels relative to actin mRNA levels. These are representative of two separate experiments.

transduction inhibitors were used. H7 is a nonspecific general kinase inhibitor that inhibits both PKC and PKA [21-23]. PMA is a specific inhibitor of PKC [24,25]. W7 is a specific inhibitor of CAM-PK [26] and was used as a control for an inhibitor, which did not affect PKC- or PKA-associated signal transduction. Before monitoring the effect of the above inhibitors on *L. donovani* infection levels, it was first necessary to verify what effect these inhibitors had on signal transduction in the BMM. PMA inhibited c-*fos* expression in response to PKC stimulation with OAG (Fig. 4A) but not in response to CT (Fig. 4B). This demonstrated that PMA impaired PKC but not PKA-associated signal transduction in the BMM. In contrast, H7 impaired c-*fos* expression in response to CT and LPS (Fig. 4C), verifying that H7 impaired both PKC- and PKA-associated signal transduction. Finally, W7 did not impair either CT- or LPS-induced c-*fos* expression (Fig. 4D), verifying that it did not inhibit either PKC- or PKA-associated signal transduction. Using the above assay, commercially available specific inhibitors of PKA, such as HA1004 or Rp-cAMP, had no inhibitory effect on the PKA-associated signal transduction. Therefore, no subsequent data are presented on the effect of these inhibitors on *L. donovani* infection levels.

To monitor the effect of the above inhibitors on *L. donovani* infection levels, H7 and W7 were added to macrophages 15 min prior to infection (20:1 amastigotes per BMM for 3 h) and were present during the 3 h infection period and the subsequent 72 h period. PMA was added overnight prior to infection and was also maintained throughout the course of the experiment. Macrophages that were treated with H7 or PMA demonstrated an increase in the initial uptake (3 h) of amastigotes as compared to

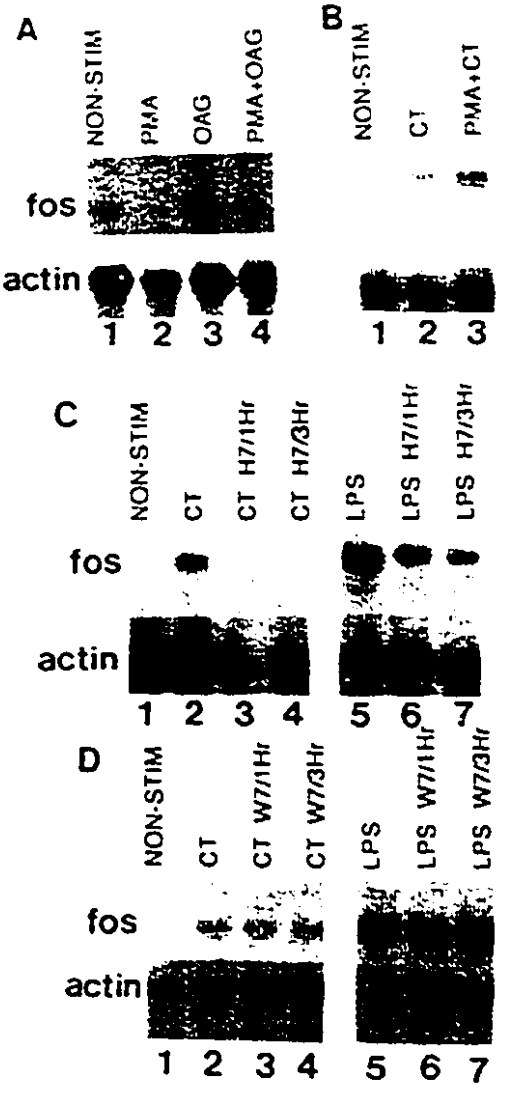

Fig. 4. Effect of protein kinase inhibitors on signal transduction in BMM. (A) Macrophages untreated *(lanes 1,3)* or treated with PMA (100 ng/ml) for 18 h *(lanes 2,4)* were stimulated with 100 uM OAG *(lanes 3,4)*. (B) Macrophages untreated *(lanes 1,2)* or treated with PMA for 18 h *(lane 3)* were then stimulated with 10 ng/ml CT *(lanes 2,3)*. (C) Macrophages untreated *(lanes 1,2,5)*, treated with H7 (20 uM) for 1 h *(lanes 3,6)*, or treated with H7 (20 uM) for 3 h *(lanes 4,7)* were stimulated with CT *(lanes 2-4)* or 100 ng/ml LPS *(lanes 5-7)*. (D) Macrophages untreated *(lanes 1,2,5)*, treated with W7 (20 uM) for 1 h *(lanes 3,6)*, or treated with W7 (20 uM) for 3 h *(lanes 4,7)* were stimulated with CT *(lanes 2-4)* or LPS *(lanes 5-7)*. After macrophage stimulation, RNA harvesting and Northern blot analysis for c-*fos* and actin was carried out as described in Materials and Methods. These are representative data of at least two seperate experiments with each inhibitor.

control non-treated macrophages (Fig. 5). In comparison, treatment of macrophages with W7 did not result in differences in the level of infection at the initial 3 h time interval as compared with non-treated macrophages. After 24 h, the most dramatic change occurred in the W7 treated macrophages, which was the only group to show a reduced level of infection, and this phenomenon continued over the three day period. From 48 to 72 h, the cells treated with H7 and PMA contained significantly more amastigotes than did nontreated control cells. Although there were more amastigotes in the H7-treated cells than in the PMA-treated cells at the 7h time interval, this difference was not significant ($p>0.05$). Treatment of normal uninfected macrophages with the above concentration of inhibitors over the same period of time did not result in a loss of macrophage viability as determined by trypan blue exclusion (data not shown). Cells treated with H7 or PMA had similar levels of infection even though H7 inhibited both PKC- and PKA-mediated signal transduction, whereas PMA only inhibited PKC-mediated signal transduction. This was particularly evident early in the infection process at the 3 h time interval after inoculation with 20:1 amastigotes/macrophage. At this time, we report a significant increase in macrophage infection in both H7- and PMA-treated macrophages. It is noteworthy that, in a previous study, control and PMA-treated macrophages had similar levels of infection 2 h after inoculation with 10:1 amastigotes [13], but that at later time intervals PMA-treated cells had higher levels of infection similar to the present study. The differences at the early time intervals are likely the result of the low level of infection (50 amastigotes/100 macrophages) at the 2 h time interval in the previous study, as compared with the high level of infection (550 amastigotes/100 macrophages) at the

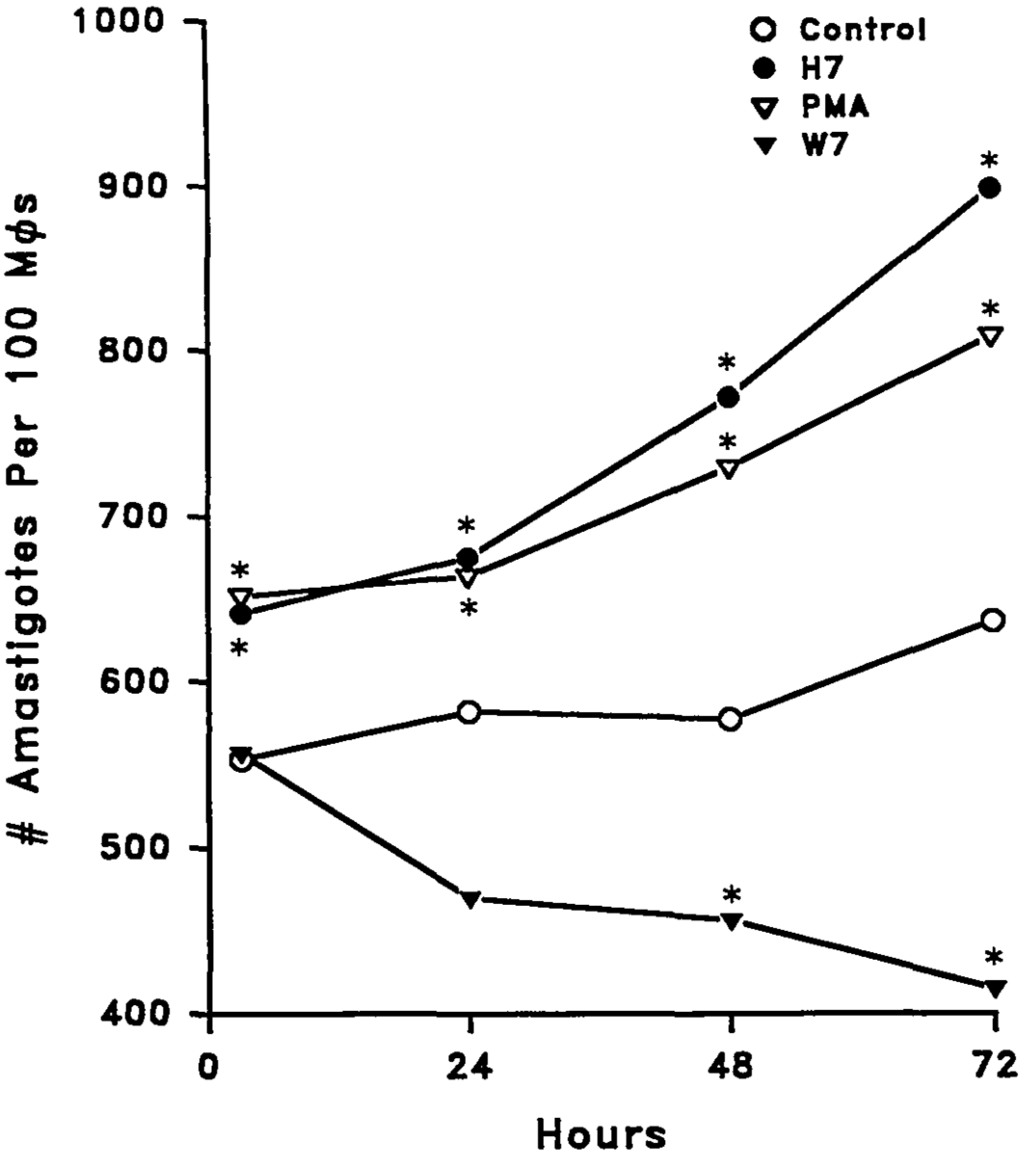

Fig. 5. Effect of protein kinase inhibitors on *L. donovani* infection levels. Macrophages were treated with 20 uM of H7 or W7 for 15 min (or 100 ng/ml PMA for 18 h) prior to infection with *L. donovani* amastigotes, and inhibitors were maintained over the course of the experiment. Infection levels were monitored 3, 24, 48 and 72 h after contact with amastigotes (20:1). Note that the first set of data points on the x-axis represent the 3 h time interval. Each point represents an average of three samples. Standard deviation values ranged from 2 to 30/time interval (analysis of variance by Dunnett's mean variance, * $p < .05$ compared with control). These are representative data of four similar analyses.

3h time interval observed in the present study.

These data argue that the inhibition of PKC, and not PKA, was predominantly responsible for the increased levels of infection. It is, however, not possible to rule out the possibility that impairment of other kinases may contribute to higher infection levels at the 72 h time interval. In direct contrast, impairment of CaM-PK caused a marked reduction in the level of infection. Taken together, this experiment demonstrated that it was possible to modulate the infection levels up or down depending on which kinase inhibitors were used, and that impairment of PKC in quiescent macrophages makes these cells more susceptible to infection by *L. donovani*.

The data presented in Figure 5 also demonstrated that W7 reduced the level of infection. To investigate the mechanism by which W7 had this effect, we examined the possibility that W7 was selectively toxic to the parasite. The proliferation of *L. donovani* promastigotes in culture was monitored in the presence of H7, PMA, and W7 for 9 days. As shown in Figure 6, W7 inhibited proliferation of *L. donovani*, whereas H7 and PMA demonstrated no such effect. These data suggest that W7 caused a reduction in the level of macrophage infection by virtue of its selective inhibitory activity on the parasite.

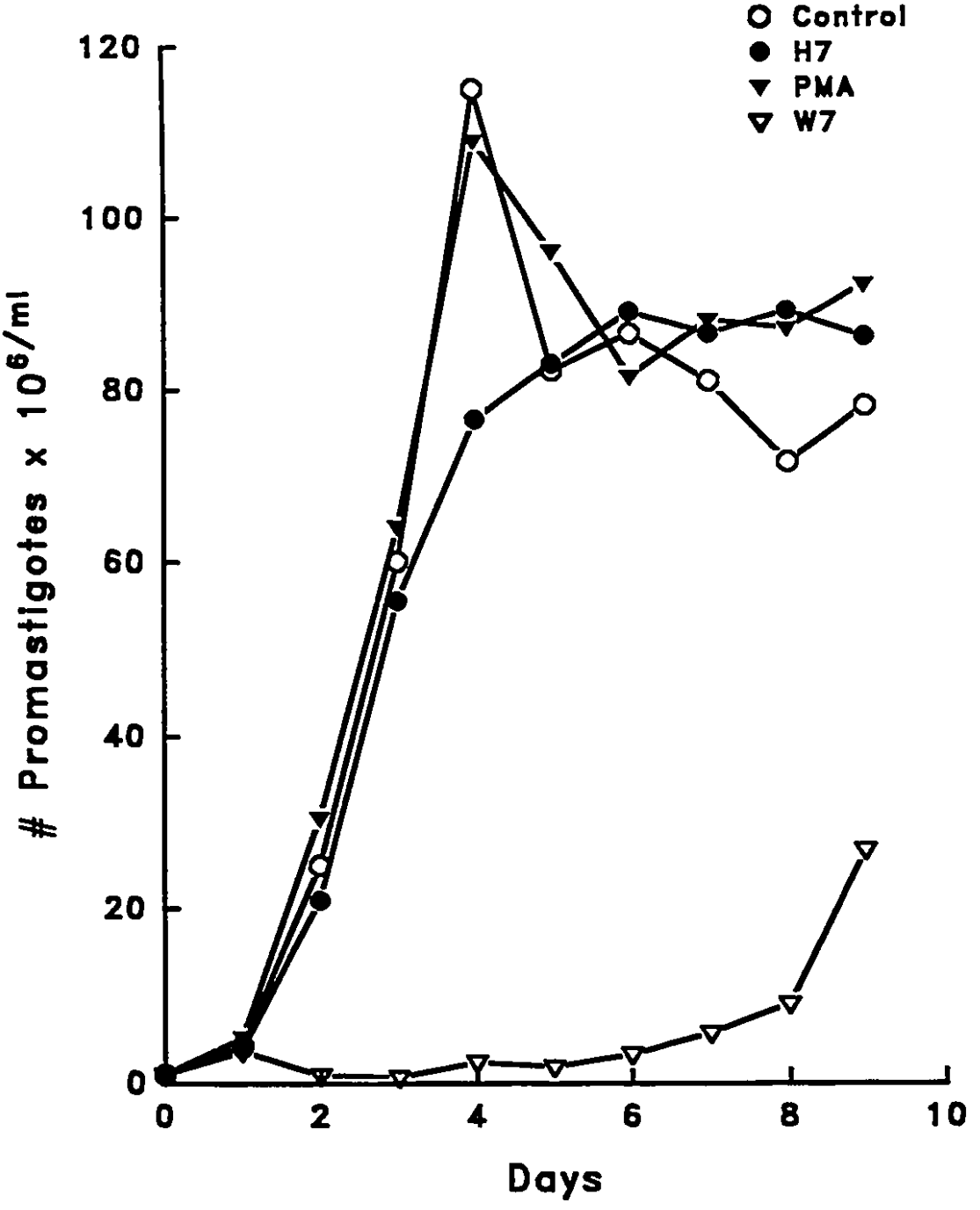

Fig. 6. Effect of protein kinase inhibitors on *L. donovani* promastigote proliferation in culture. Promastigote cultures were treated with 20 uM of H7 or W7 and 100 ng/ml PMA, and the presence of inhibitors was maintained throughout the experiment. Promastigote numbers were monitored daily, and each point represents the average of three samples. These are representative data of three similar analyses.

DISCUSSION:

In the present study, we examined the specificity with which *L. donovani* impairs signal transduction to the nucleus and the biological implication of this activity. Three major observations are reported here. First, *L. donovani* amastigotes and promastigotes impaired PKC-, but not PKA-mediated signal transduction to the nucleus. Second, impaired PKC signal transduction was biologically relevant, as impairment of this cellular pathway appeared to be predominantly responsible for increasing infection levels. This was especially evident early during the infection process. Third, macrophages treated with an inhibitor of CaM-PK carried a lower parasite burden than did control cells, and this effect was likely the result of the selective toxic activity of this inhibitor toward *L. donovani*.

It has been previously reported by our lab that the major surface molecule of *Leishmania* promastigotes, LPG, was capable of impairing PKC- but not PKA-mediated gene expression in macrophages [8], and that LPG would inhibit PKC activity in intact cells [13]. However, as a result of recent evidence that LPG was not detectable in amastigotes of *L. donovani* [14], it was necessary to determine whether the live parasite (promastigotes and amastigotes) similarly selectively impaired signal transduction. As demonstrated here, both promastigotes and amastigotes had a similar effect on signal transduction as did the promastigote-derived LPG molecule. The most pronounced inhibition of PKC-mediated c-*fos* gene expression occurred in macrophages placed in contact with promastigotes or amastigotes for 1 h, with virtually no inhibitory activity

toward PKA mediated gene expression. During this initial 1 h contact period, there may be a number of as yet undetermined molecular perturbations in the macrophage cell that may result in the translocation of PKC from the cytosol to the membrane where its activity may have been inhibited. In the case of the promastigotes, we have data suggesting that this inhibition may have been achieved by the LPG molecule [8,13]. Furthermore, it has been shown that LPG can rapidly be transferred from the promastigote to coat the surface of the macrophage membrane [27]. In the case of amastigotes, other surface glycosylphosphotidylinositol molecules have been shown to inhibit PKC *in vitro* [28], and these may have been responsible for impairing PKC-mediated gene expression in this study.

These data also suggest that impairment of PKC early during this host-parasite interaction may be important in the establishment of infection by this protozoan. This is consistent with the data showing that at the 3h interval, cells treated with H7 or PMA had significantly higher infection levels than did control or W7 treated cells. Moreover, we demonstrate that at the 3h time interval, the level of infection was the same in H7- and PMA-treated cells, arguing that impairment of PKC was responsible for the increased infection levels rather than impairment of other protein kinases such as PKA, which was also inhibited by H7 in these cells. However, the specific isoforms of PKC which were inhibited by PMA treatment were not determined in this study. Although impairment of PKC enhanced infection levels and may be necessary for infection, this is not sufficient to induce infection as noninfective promastigotes also impair PKC-mediated signal transduction. This implies that other macrophage and/or parasite factors may also be

necessary for infection. Finally, Figure 4 showed that PMA did not completely inhibit OAG-induced c-*fos* expression, suggesting that PMA may not have inhibited all the PKC species in these BMM. Nevertheless, the data in Figure 5 argue that the PKC species that were impaired by PMA were involved in resisting infection with *L. donovani*.

Macrophages infected for 18 h were also impaired with respect to PKC-mediated c-*fos* gene expression, but this effect was not as pronounced as in the cells treated with the parasite for 1 h. Nevertheless, long-term impairment of PKC with H7 and PMA also favoured the parasite because at the two and three day time intervals after amastigote infection, there was a significantly greater level of parasite infection in the PKC-inhibited cells than the control cells. Although there is evidence to suggest that the promastigote LPG molecule may be the major inhibitor of PKC-mediated signal transduction [13], it is difficult to speculate about the molecular basis for amastigote-mediated down-regulated PKC signal transduction in infected cells, as LPG is undetectable on *L. donovani* amastigotes [14]. Further studies with purified amastigote-derived molecules may provide insight into this phenomenon.

The gene expression analysis revealed that *L. donovani* had little impact on PKA-mediated gene expression. Recently, it has been reported that *L. donovani* infection did not alter cAMP levels in macrophages, which further supports the view that the cAMP-PKA pathway is unaffected in infected cells [29]. This suggests that *L. donovani* has not developed mechanisms to impair the PKA-mediated signal transduction pathway. Taken together, these observations argue that *L. donovani* has selectively evolved PKC inhibitory mechanisms because this benefits the parasite by enhancing the level of

infection.

Of particular interest in this study, we observed that the CaM-PK inhibitor reduced the level of *L. donovani* infection in macrophages, and this may have practical applications. This was most likely the result of the selective toxic effect of this molecule against the parasite as promastigote proliferation was dramatically reduced by W7, but not H7 or PMA. There is currently little information on signal transduction in *Leishmania*, and these data suggest that CaM-PK-mediated biochemical processes are essential to the parasite. It was also interesting to note that the PKC inhibitors H7 and PMA had no effect on promastigote proliferation in culture. This is consistent with the idea that *Leishmania* produces its own PKC antagonist and thus must also be resistant to PKC inhibitors.

In conclusion, our results show that *L. donovani* has evolved to selectively impair host cell biochemical processes involved in transmitting information to the nucleus and such activity enhances the level of infection with this protozoan. In particular, impairment of PKC provides an advantage for the parasite over the macrophage cell. In comparison, selective impairment of CaM-PK provided an advantage for the macrophage cell, as this was more toxic to the parasite than the host cell. Selective impairment of signal transduction may therefore represent a new strategy for treating *Leishmania* infection.

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CONNECTING STATEMENT TO MANUSCRIPT II

In the first manuscript, it was demonstrated that *L. donovani* selectively impairs macrophage signal transduction to the nucleus, and this impairment was biologically relevant with respect to establishment of infection by the parasite. This study, and previous studies on the mechanism of intracellular survival of *Leishmania*, have focused primarily on the negative effect which this parasite has on macrophage function. To fully define the molecular basis of *Leishmania* infection, both negative and positive effects of infection on the host must be investigated. However, it has never been established whether *Leishmania* enhances macrophages in any way. Therefore, I have undertaken to determine whether *L. donovani* enhances any biological aspects of macrophages.

Throughout our earlier studies, we observed that there were consistently more infected cells than relevant non-infected cells, but this was never investigated. This represented a potential novel discovery dealing with intracellular protozoan infections which has been investigated in the next two manuscripts. While it has been known for some time that intracellular infections with some viruses can stimulate the proliferation of the host cell, this question has never been addressed in a protozoan system. The ensuing manuscript delineates this novel enhancement of macrophage viability by *Leishmania* infection, and discusses the implications of this positive influence by the parasite on its host cell.

MANUSCRIPT II

LEISHMANIA DONOVANI INFECTION ENHANCES MACROPHAGE VIABILITY IN THE ABSENCE OF EXOGENOUS GROWTH FACTOR[1]

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FOOTNOTES:

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2. Abbreviations:

BMM, bone marrow derived macrophage; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LCM, L-929 cell-conditioned medium; M-CSF, macrophage colony-stimulating factor; CHX, cycloheximide; MTT, (3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide;thiazolyl blue); GM-CSF, granulocyte-macrophage colony-stimulating factor; TNFα, tumor necrosis factor alpha; TGF-ß, Transforming growth factor beta.

ABSTRACT:

Bone marrow-derived macrophages rapidly die in the absence of macrophage growth factor (M-CSF).[2] However, as demonstrated here, bone marrow-derived macrophages infected with *Leishmania donovani* exhibit increased viability in the absence of exogenous growth factor. Forty-eight hours after inoculation with promastigotes or amastigotes, infected cell cultures contained 180% and 95% more cells, respectively, than control cultures. This effect was specific to *Leishmania* infection, as uptake of latex beads or avirulent promastigotes by macrophages did not enhance cell viability. *L. donovani*-infected macrophages also displayed increased phagocytic capacity, as compared with control macrophages and macrophages grown continuously in M-CSF-containing medium. Supernatants collected from infected cells elaborated a factor(s) that enhanced macrophage viability but did not stimulate macrophage DNA synthesis. This activity of *L. donovani*-infected cell-conditioned medium could be abrogated by preincubation of macrophages with cycloheximide before inoculation with the parasite, implying that macrophage protein synthesis is required for the elaboration of this factor(s).

Key Words: *Leishmania*, macrophage viability

INTRODUCTION:

Mononuclear phagocytes play a critical role in host immune responses to microbial infection and are a major source of inflammatory and growth cytokines. Within its mammalian host, *Leishmania donovani* survives by intracellular parasitization of macrophages, the exclusive host cells for this protozoan. Resolution of leishmanial infection requires the development of effective cell-mediated immunity culminating in the activation of macrophages to a microbicidal state [1]. However, in humans and mice, *L. donovani* causes persistent and potentially fatal visceral infections, pointing to a breakdown of macrophage function and macrophage-T cell interaction in parasite-specific immunity [2-9]. For example, it has been established that *Leishmania*-infected macrophages are inhibited in their ability to express macrophage activation-associated functions [2-9]. However, it has not been established whether *L. donovani* enhances macrophages in any way, such as macrophage viability and survival in the absence of macrophage growth factor.

The mechanisms involved in monocyte/macrophage survival, accumulation, and death are poorly understood. Studies have identified several cytokines and exogenous stimuli that may regulate monocyte/macrophage viability [10,11]. Culture of macrophages derived from bone marrow exudates requires the presence of macrophage colony-stimulating factor (M-CSF) for sustained growth and differentiation [12,13]. Upon removal of this growth factor, bone marrow-derived macrophages (BMMs) undergo

a rapid decline in cell viability resulting in the death of approximately 70% of the population within 48 h [11]. In the present work, we show that macrophages infected with *L. donovani* elaborate a soluble factor into the cell supernatant that increases host cell viability in the absence of exogenous growth factor. Elaboration of such a factor(s) from infected macrophages may play an important role in the infection process. In addition, this may represent a means of increasing the number of circulating host cells available for infection.

MATERIALS AND METHODS:

Reagents

RPMI 1640 with L-glutamine, D-MEM with L-glutamine, and Hanks' balanced salt solution (HBSS) without calcium and magnesium were purchased from Gibco/BRL Life Technologies (Burlington, Ont.). Low-endotoxin fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT). Polystyrene latex beads (1.1μm diameter), cycloheximide and MTT were purchased from Sigma Chemical Co. (St. Louis, Mo). [^{3}H]Thymidine was purchased from ICN Radiochemicals (Mississauga, Ont.).

Leishmania

Two strains of *L. donovani* were used in this study, the 1S2D and the LV9 strains. Amastigotes were isolated from the spleens of infected Syrian hamsters as previously described [14]. Promastigotes of *L. donovani* were derived through amastigote culture at 27°C, 5% CO_2 in modified D-MEM supplemented with 10% heat-inactivated FCS [15]. These organisms were maintained for a maximum of three passages *in vitro* and were used when they had reached the stationary phase of growth [16]. Avirulent promastigotes of the 1S2D strain of *L. donovani* that are able to invade macrophages but cannot sustain infection were cultured over several years as described above. The long-term *in vitro* cultivation of this 1S2D promastigote culture is the most likely cause of its avirulence.

Bone marrow-derived macrophages

Six- to eight-week-old female BALB/c mice (Charles River, St. Constant, Que.) were used as a source of bone marrow cells for all studies. BMMs were prepared as previously described [5,6,8,9]. Femurs were flushed with RPMI 1640 complete medium containing 10% heat-inactivated FCS, 10 Mm HEPES and 100 U/ml each of penicillin and streptomycin. Bone marrow cells were incubated for 18 h in tissue culture dishes (Falcon 3003) in the presence of 10% L929 cell-conditioned medium (LCM) as a source of M-CSF to separate adherent differentiated cells. Nonadherent, immature cells were removed and cultured for an additional 4 days in 10% LCM to induce macrophage differentiation, and this was carried out in polystyrene culture dishes (Falcon 1029), which are weakly adherent for macrophages. The resulting macrophage population was made quiescent as follows; 18 h before use, nonadherent and loosely adherent cells were washed in ice-cold HBSS without calcium and magnesium and detached from dishes using a cell scraper. BMMs were resuspended in RPMI complete medium in the absence of exogenous LCM. Viability was determined by trypan blue exclusion and counting live cells with a hemacytometer. Viability was also determined in some instances by the MTT assay described below, which gave comparable results to the assay described above.

Infection of BMMs with *Leishmania*

Quiescent BMMs (1.25×10^6 cells/ml) were plated in 60-mm polystyrene dishes, which are weakly adherent for macrophages (Falcon 1008), in a total volume of 2 ml. On day 0, amastigotes, infective promastigotes, noninfective promastigotes, or latex beads

were added to quiescent BMMs at a ratio of 7:1. Adherent and nonadherent BMMs were harvested daily in the following manner. Nonadherent cells were collected from supernatants and loosely adherent BMMs washed in ice-cold HBSS without calcium and magnesium and detached using a cell scraper. In this manner, both adherent and nonadherent BMMs were counted. Cell counts were determined by trypan blue exclusion using a hemacytometer (0.1mm). Triplicate samples were taken for each group. The percentage of infected cells was routinely >95% after 3 h, with four to six amastigotes per macrophage as determined by cytospin and Giemsa staining.

Assay of phagocytic capacity

The phagocytic capacity of control and promastigote-infected BMMs that had been in the absence of exogenous growth factor for 48 h was assayed as a measure of cell viability as previously described [17]. Briefly, polystyrene latex beads (1.1μm in diameter) were washed four times in RPMI complete medium, centrifuged at 16,000*g* for 15 min, and resuspended at 1 x 10^9 beads/ml. Latex beads were added to equal numbers of BMMs at 20:1 for 2 h, after which cells were washed three times in RPMI complete medium and incubated for an additional hour to ensure phagocytosis of remaining beads. Cells were harvested and cytospin samples were stained by Giemsa stain. Phagocytic capacity was measured by the percentage of the BMM population that was actively phagocytic and by the number of latex beads per macrophages. As an additional comparison, latex beads were added to BMMs grown continuously in LCM-containing medium.

L. donovani-infected BMM supernatants

Quiescent BMMs (1 x 10^6 cells/ml) were infected with promastigotes at a ratio of 10:1 promastigotes per macrophage for 3 h. BMMs were washed three times to remove excess parasites and resuspended in RPMI complete medium. Giemsa staining was used to monitor the level of infection, which was routinely 4-6 amastigotes/macrophage. Infected and noninfected cell supernatants were collected 3, 6, 12 and 24 h after inoculation, centrifuged at 100*g*, and filtered before use. In this manner, the 3 h postinoculation time represented the zero time point following infection. Supernatants were assayed for their ability to enhance macrophage viability at 75% strength by addition to quiescent BMMs prepared as described above. Cell viability counts were performed by trypan blue exclusion as described.

[^{3}H]Thymidine incorporation assay

DNA synthesis of BMMs was measured by the incorporation of [^{3}H]thymidine as previously described [18]. Quiescent BMMs were plated at 5 x 10^5 macrophages in 0.5 mL in 24-well plates. Control supernatants from uninfected macrophages and supernatants from macrophages infected for 24 h with either the 1S2D or the LV9 strain of *L. donovani* were added at 75% v/v to quiescent BMMs for 48 h. As an additional comparison, macrophages were treated with 20% LCM to measure the effect of M-CSF on cell proliferation. DNA synthesis was assayed by labelling BMMs with [^{3}H]thymidine (3 *u*Ci per plate, specific activity 53 Ci/mmol) during the last 24 h of culture. The cells were then washed three times with phosphate-buffered saline and 1 ml of 0.2 N NaOH

containing 40 *ug*/ml of salmon sperm carrier DNA was added to each plate. The cultures were harvested by filtration through Whatman GF/C glass fiber filters and washed with 10% trichloroacetic acid. [^{3}H]thymidine incorporation was determined by liquid scintillation spectrometry.

MTT viability assay

Macrophage viability was also measured using the MTT assay, which is based on the ability of live cells to convert water-soluble tetrazolium salt to insoluble formazan [19]. Only active mitochondrial dehydrogenases from living cells cause this conversion, and the purple precipitate can be dissolved in isopropanol and quantified spectrophotometrically. This assay was performed according to the recommendation of the manufacturer, which is a modification of the original protocol for measuring cell proliferation [20]. Briefly, BMMs were plated at 5 x 10^4 cells in a total volume of 0.2 ml in 96-well plates. Control supernatants from uninfected macrophages or supernatants from macrophages infected for 24 h with either the 1S2D or the LV9 strain of *L. donovani* were added at 75% v/v to quiescent BMMs for 48 h. As an additional comparison, macrophages were treated with 20% LCM to measure the effect of M-CSF on macrophage viability. MTT (5 mg/ml) was added at 1/10 total volume for the last 4 h of culture. The culture media was removed and MTT-formazan was solubilized by adding acidic isopropanol. Formazan was measured spectrophotometrically at 570 nm.

Cycloheximide pretreatment of BMMs

Quiescent BMMs in suspension (2 x 10^6 cells/ml) were treated with 18 mM cycloheximide for 2 h prior to infection as previously described [21]. Treated BMMs were washed four times with RPMI complete medium to remove any exogenous cycloheximide and inoculated with 10:1 promastigotes for 3 h. Cell supernatants were collected after 6 h and assayed for their ability to enhance macrophage viability as described above. The infection levels of cycloheximide-treated macrophages was the same as in non-cycloheximide-treated macrophages.

RESULTS:

L. donovani infection specifically increases BMM viability

The viability of BMMs inoculated with either *L. donovani* promastigotes or polystyrene latex beads at a ratio of 7:1 was monitored 3, 24, 48 and 72 h after inoculation. As demonstrated in Figure 1A, in the absence of exogenous growth factor macrophages infected with *L. donovani* promastigotes exhibited a marked increase in viability over control noninfected macrophages. This augmentation of host cell viability was apparent 24 h after infection (46% more viable cells) and was maintained throughout the course of the study. A similar pattern was apparent in BMMs infected with amastigotes (7:1) of *L. donovani* (Fig. 1B), although to a lesser degree than in those infected with promastigotes. Although amastigotes consistently enhanced BMM viability, promastigotes were more efficient; therefore, subsequent analyses were performed using promastigotes. This effect of increased BMM viability could not be duplicated by the uptake of inert latex beads (Fig. 1A), implying that this was a parasite-mediated event and not a general result of phagocytosis. Photographs taken of BMMs infected with *L. donovani* promastigotes 18 h after inoculation (Fig. 2B) clearly show greater cell density in infected cell cultures compared with control BMMs cultures (Fig. 2A). Infected cell cultures exhibited increased adherence and cell aggregation, as well as a greater percentage of light refractile cells. It is clear from Figure 2 that the infected cell population was more healthy than the noninfected cell population. The effects of augmented host cell viability in *L. donovani*-infected macrophages shown in Figures 1

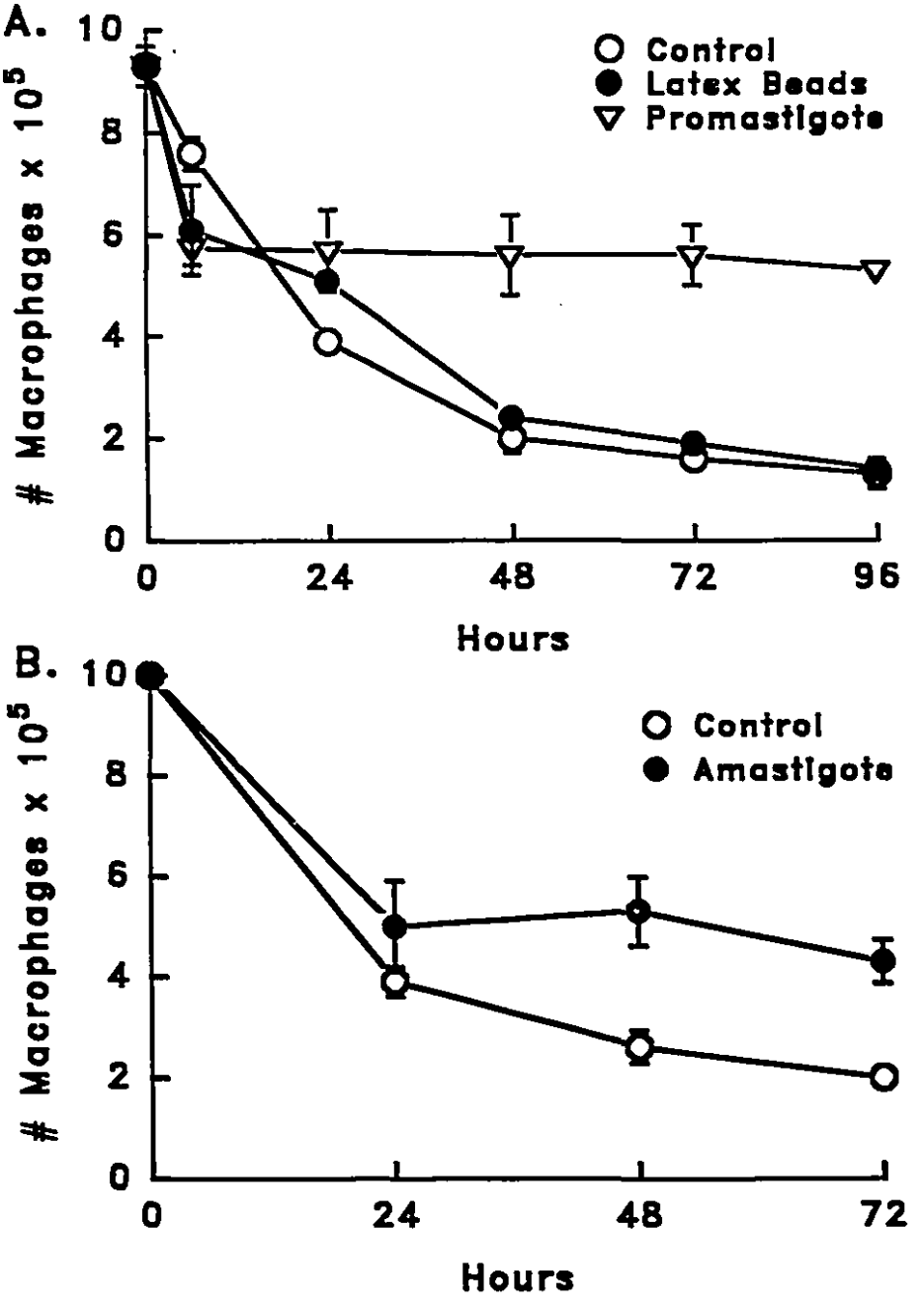
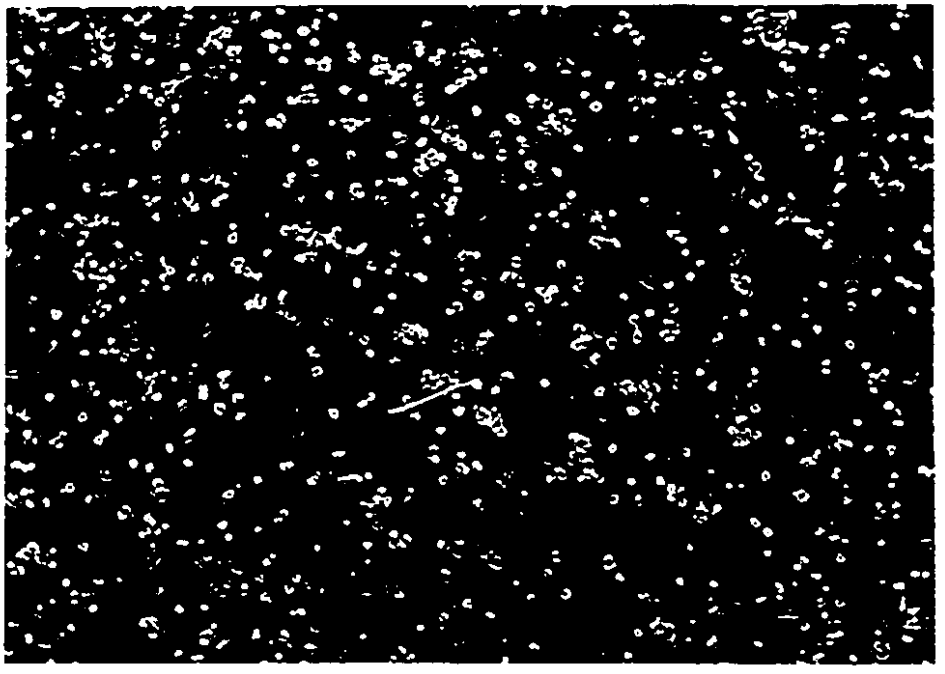


Fig. 1. Viability of BMMs is specifically increased by *L. donovani* promastigote or amastigote infection but not by phagocytosis of latex beads. (A) Quiescent BMMs were inoculated on day 0 with 7:1 promastigotes/Mϕ (▲) or 10:1 latex beads/Mϕ (●) or cultured in control medium with no growth factor (○). (B) BMMs were inoculated on day 0 with 7:1 amastigotes/Mϕ (●) or cultured in control medium with no growth factor (○). BMMs were harvested and counted daily. Values are expressed as the mean number of BMMs/ml (± standard deviation) of triplicate samples. Statistical analysis was performed by the Bonferroni t-test (* significantly different from control, $p<0.05$). These data are representative of five experiments.

(A)



(B)

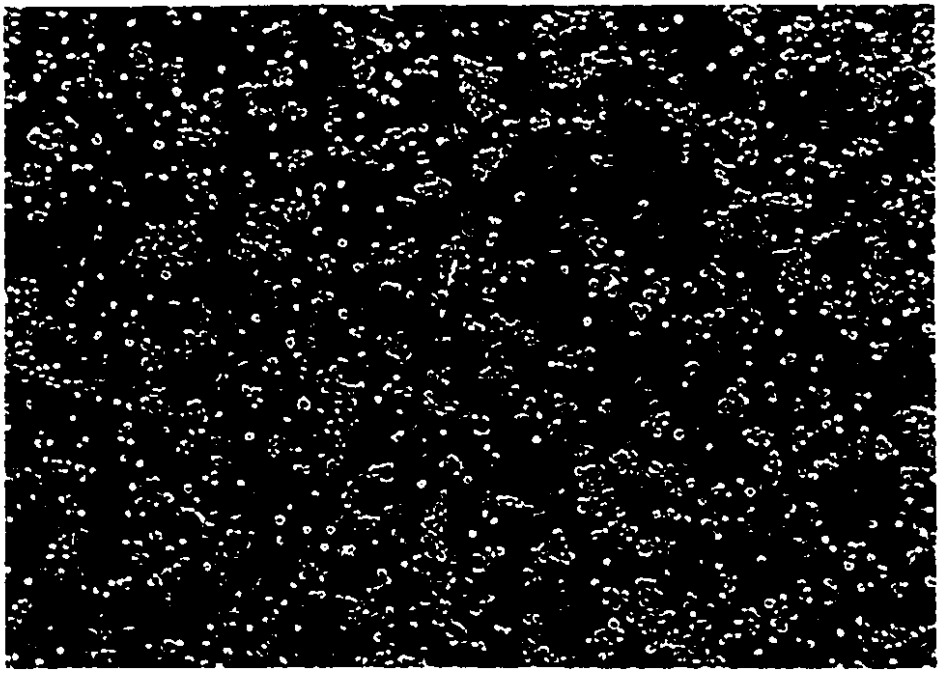


Fig. 2. Photographs (magnification x150) of *L. donovani*-infected cell cultures taken 18 h after infection (B) compared to noninfected cell cultures (A). BMMs were made quiescent by the removal of LCM prior to infection with promastigotes as described in Materials and Methods.

and 2 were representative of cell populations that were 95% infected and contained 4-6 amastigotes per macrophage. This effect was also apparent at lower levels of infection, although, predictably, it was not as dramatic. In addition, starting with low infection levels, the percentage of infected macrophages did not increase significantly with time, implying that both infected and non-infected macrophages exhibited enhanced viability (data not shown).

To determine whether sustained infection of macrophages by *L. donovani* is required for the increase in host cell viability, quiescent BMMs were inoculated with an infective promastigote strain or an avirulent promastigote strain that is internalized by the macrophage but cannot maintain an active intracellular infection. Both the virulent and avirulent 1S2D strains were cultured in exactly the same manner before infection. Only infection of BMMs by the infective promastigote strain influenced macrophage viability after 48 h (Fig. 3), confirming that uptake alone of the parasite was not sufficient to mediate this effect and that active infection was required.

One well-defined activity of macrophages is phagocytosis, and this characteristic is central to the scavenging role of this cell. To further characterize the ability of *L. donovani* to enhance macrophage viability, the phagocytic capacity of control and infected BMMs that had been in the absence of exogenous growth factor for 48 h was measured by their ability to take up latex beads added to the culture medium. As an additional comparison, the phagocytic capacity of BMMs grown continuously in growth factor was also measured. Macrophages infected with *L. donovani* exhibited both an increase in the percentage of cells which were actively phagocytic and a greater number of latex beads

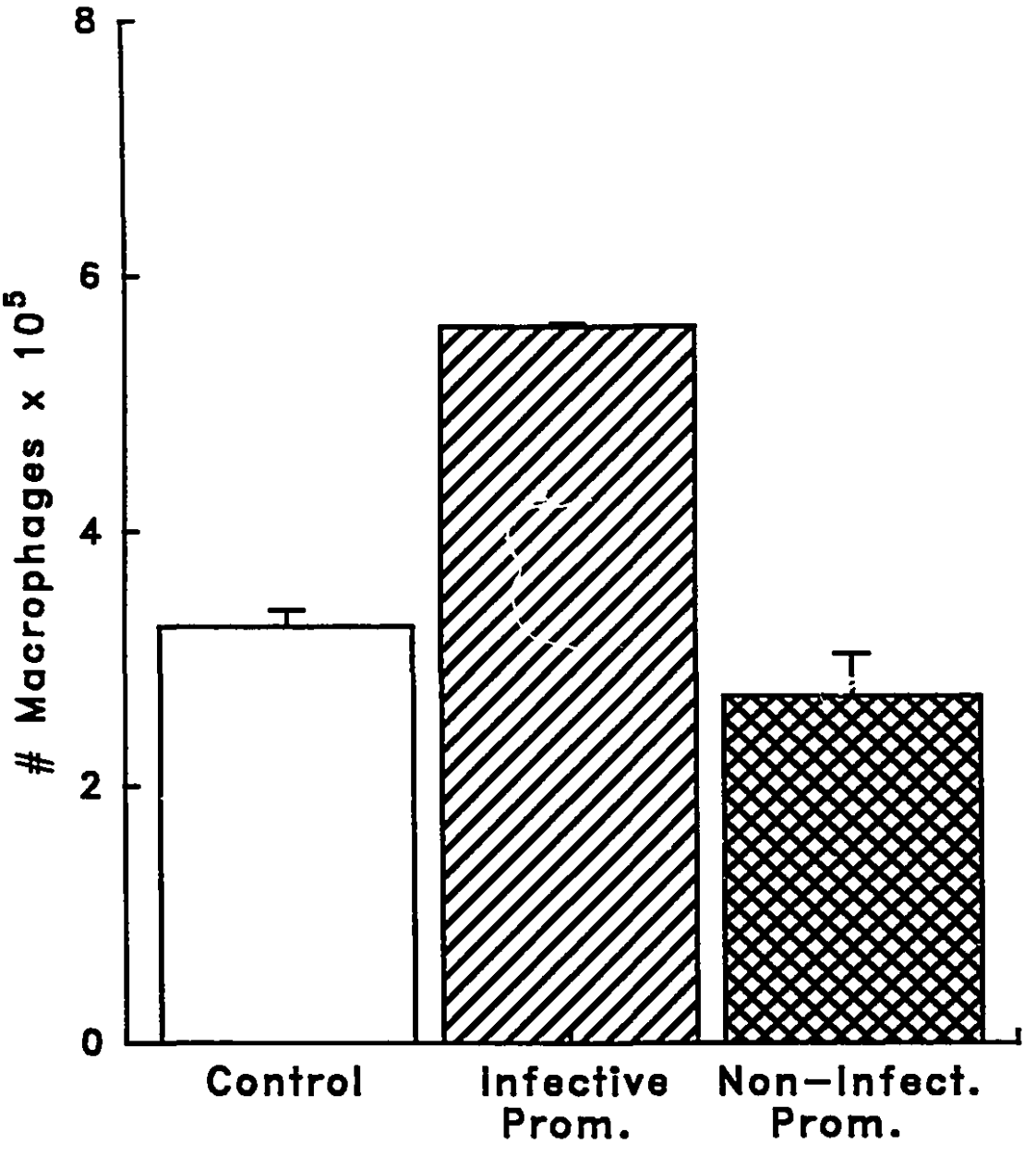


Fig. 3. Sustained infection of BMMs by *L. donovani* is required for increased viability of cells. BMMs were inoculated with infective or noninfective promastigotes and cells were harvested and counted 48 h after inoculation. At the time of collection, BMMs inoculated with infective promastigotes were 85% infected and contained an average of 4 amastigotes per macrophage. BMMs inoculated with the noninfective promastigote strain had cleared the infection. Values represent the mean number of BMMs per ml (± standard deviation) of triplicate samples. Statistical analysis was performed by the Bonferroni t-test (* significantly different from control, $p<0.05$). These data are representative of two experiments.

per macrophage compared with control cultures or cultures grown continuously in the presence of M-CSF. After 48 h in the absence of growth factor, 74% of infected macrophages were actively phagocytic (Fig. 4A) and contained an average of 4.2 latex beads per macrophage (Fig. 4B). In comparison, 55% of control macrophages and 67% of macrophages grown continuously in M-CSF were actively phagocytic, containing 2.5 and 2.8 latex beads per macrophage, respectively. These results revealed that macrophages infected with *L. donovani* exhibited an increased ability to phagocytize latex beads compared to control cells. These data further support the argument that in the absence of macrophage growth factor, *L. donovani*-infected cells are more biologically active than noninfected cells.

Supernatants of *L. donovani*-infected macrophages sustain BMMs

Macrophages have the ability to elaborate a variety of cytokines capable of modulating the activity of macrophages and other cell types. We therefore investigated the possibility that infected macrophages may release a modulator(s) responsible for increasing macrophage viability. Supernatants from *L. donovani*-infected macrophages were monitored for their ability to sustain quiescent BMMs for 48 h in the absence of exogenous growth factor. As demonstrated in Figure 5, cell-free supernatants from macrophages as early as 3 h after inoculation exhibited viability-sustaining activity, and this activity was still detectable in infected cell supernatants at 24 h after infection. Time points earlier than 3 h were not examined, as this represented the time required for infection of BMMs. The conditioned media from infected cells maintained the viability

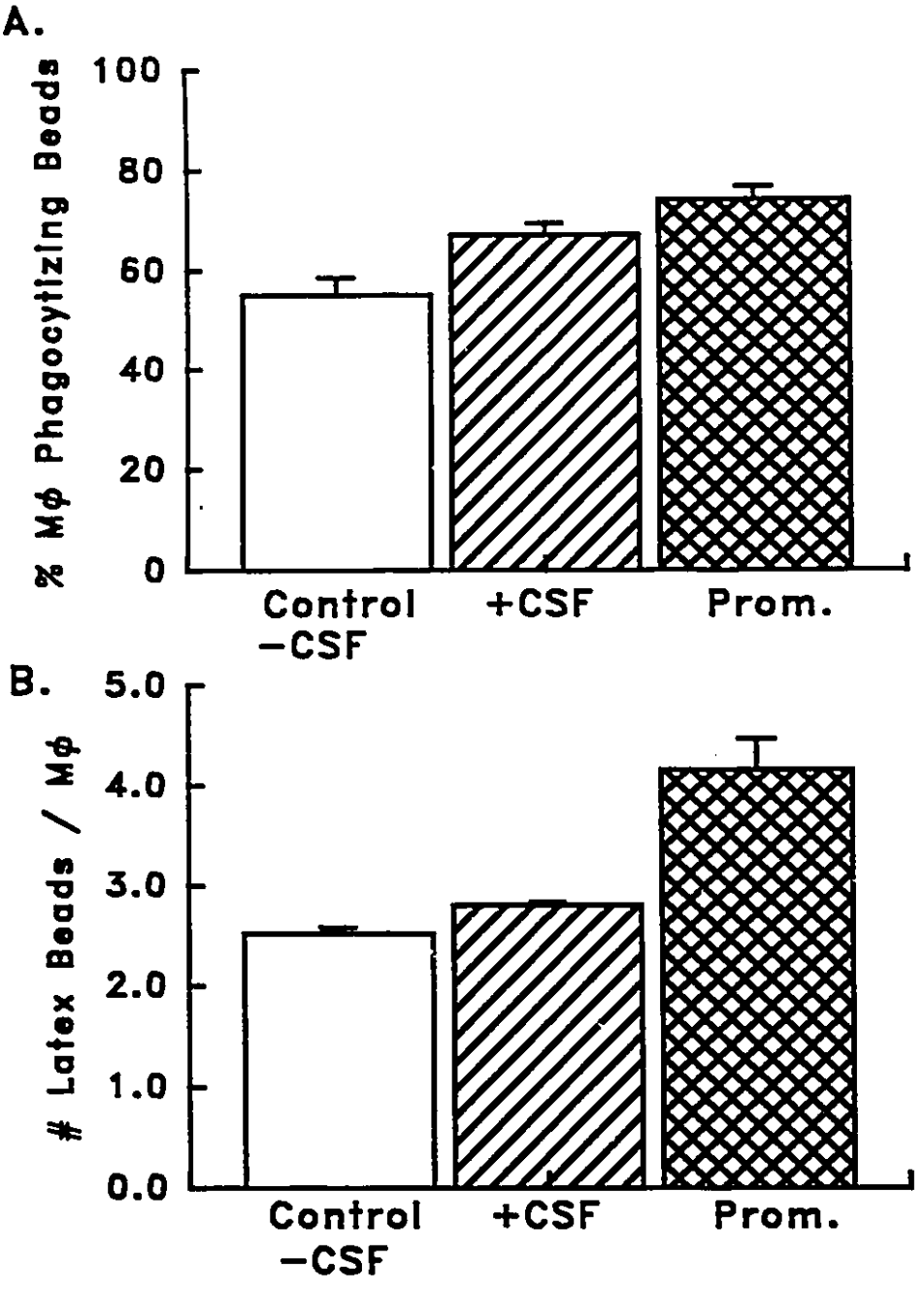


Fig. 4. Macrophages infected with *L. donovani* have increased phagocytic capacity. Control (-CSF) and promastigote-infected (Prom) macrophages maintained in the absence of growth factor for 2 days (A,B) were inoculated with latex beads (10:1 beads/Mφ) as described in Materials and Methods. As an additional comparison, latex beads were added to macrophages grown continuously in LCM (+CSF). After 3 h, cultures were washed 4X in RPMI and cytospin samples were taken. Samples were stained with Giemsa stain and scored for the percentage of macrophages containing latex beads (A) and the number of latex beads/Mφ (B). Values represent the mean (± SD) of triplicate samples. Statistical analysis was performed by the Bonferroni t-test (* significantly different from control, $p<0.05$). These data are representative of three similar experiments.

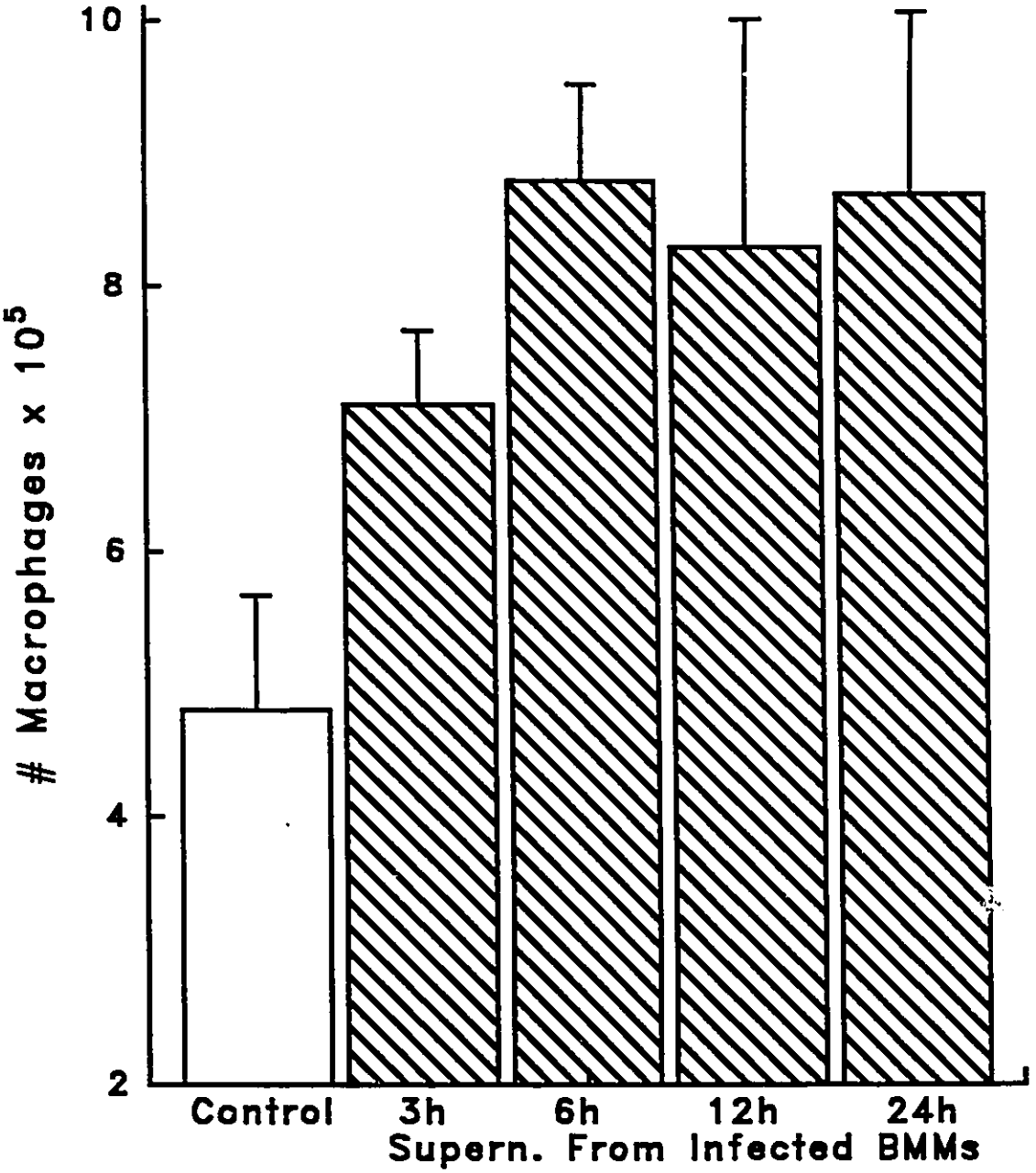


Fig. 5. Supernatants of BMMs infected with *L. donovani* promastigotes are able to enhance BMM viability in the absence of macrophage growth factor. Supernatants collected 3, 6, 12 and 24 h after infection were assayed (75% v/v) for their ability to sustain the viability of BMMs as described in Materials and Methods. The control represents the activity of supernatant from noninfected cells cultured for 24 h. Values represent the mean number of BMMs per ml (± SD) of triplicate samples. Statistical analysis was performed by the Bonferroni t-test (* significantly different from control, $p<0.05$). These data are representative of three similar experiments.

of quiescent noninfected BMMs to a level similar to that observed in infected macrophages. BMMs treated with cell supernatants taken 6 h after infection contained 83% more cells after 48 h than did BMMs cultured in control supernatants. Supernatants from noninfected macrophages did not mediate sustained viability of BMMs. These findings imply that a soluble factor was secreted by *L. donovani*-infected macrophages into the culture medium and enhanced the viability of quiescent BMMs. Supernatant from macrophages infected for 24 h with the 1S2D strain of *L. donovani* was also used to examine the effect of dilution on the viability-enhancing activity of infected cell supernatant. Dilution of infected cell supernatants resulted in reduced activity which could be titrated out at a concentration of 10% v/v (data not shown). From this dose-response curve, a concentration of 75% v/v of 24 h infected cell supernatant was selected for use in further experiments.

Supernatants of *L. donovani*-infected macrophages do not stimulate DNA synthesis

We next investigated the possibility that supernatant from infected BMMs could stimulate replication of quiescent BMMs. DNA replication was determined by measuring [^{3}H]thymidine incorporation into BMMs following treatment with supernatants from macrophages infected with either the 1S2D or the LV9 strain of *L. donovani*. The MTT cell viability assay was performed in tandem to monitor the effect of infected cell supernatants on viability of this macrophage population. As demonstrated in Figure 6, supernatants from infected macrophages did not induce DNA synthesis (Fig. 6A), but did increase cell viability (Fig. 6B). Similar results were obtained using supernatants from

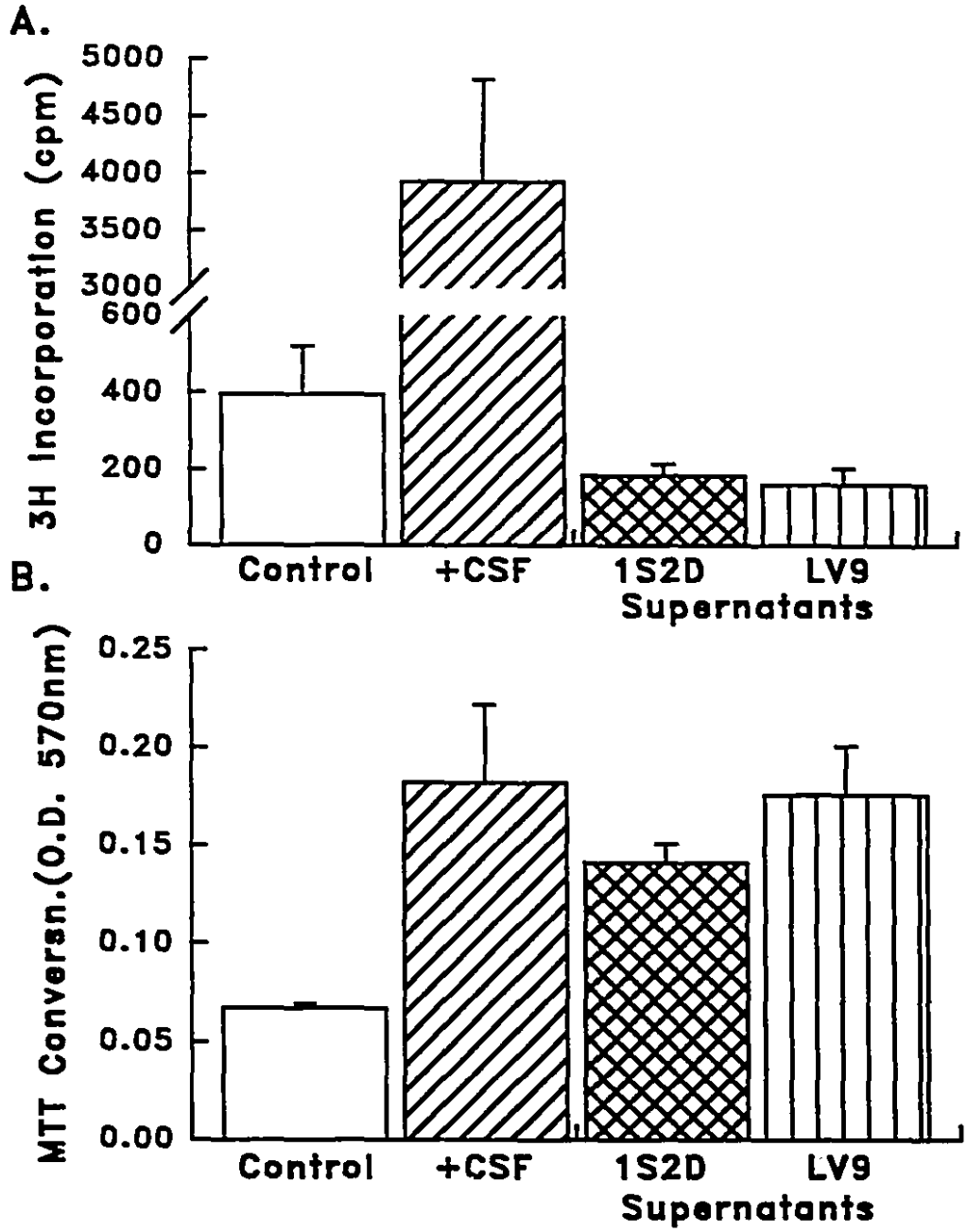


Fig. 6. Supernatants from BMMs infected with two strains of *L. donovani* promastigotes are able to enhance BMMs viability but do not stimulate DNA synthesis. (A) DNA synthesis induced by supernatants (75% v/v) collected from BMMs 24 h after infection with either the 1S2D or the LV9 strain of *L. donovani* was measured by [^{3}H]thymidine uptake as described in Materials and Methods. The control represents the activity of supernatants from noninfected cells cultured for 24 h. M-CSF-treated BMMs (+CSF) contained 20% v/v LCM. (B) Cell viability induced by supernatants (75% v/v) collected from BMMs 24 h after infection with either the 1S2D or the LV9 strain of *L. donovani* as determined by the MTT viability assay. Values represent the mean number (± SD) of triplicate samples. Statistical analysis was performed by the Bonferroni t-test (* significantly different from control, $p<0.05$).

macrophages infected with either the 1S2D or the LV9 strain of *L. donovani*. In contrast, addition of 20% M-CSF resulted in a significant increase in both DNA replication and cell viability. These data imply that the active component of *L. donovani*-infected cell supernatant is increasing macrophage viability in a manner independent of cell replication.

Elaboration of active factor into culture supernatants requires protein synthesis

To investigate whether protein synthesis was necessary for the elaboration of this factor(s), BMMs were pretreated with cycloheximide, a protein synthesis inhibitor, 2 h prior to infection with *L. donovani* promastigotes. Cycloheximide-treated and nontreated BMMs had the same level of infection, demonstrating that pretreatment with this protein synthesis inhibitor did not influence the level of infection. As demonstrated in Figure 7, supernatants from infected macrophages pretreated with cycloheximide no longer exhibited the ability to sustain BMMs. Cycloheximide pretreatment of macrophages was able to reduce the activity of these supernatants to control levels, implicating a role for macrophage protein synthesis in the elaboration of this factor by *L. donovani* infection. We cannot, however, rule out the possibility that the cycloheximide pretreatment inhibited the synthesis of a *L. donovani*-derived protein responsible for enhancing macrophage viability.

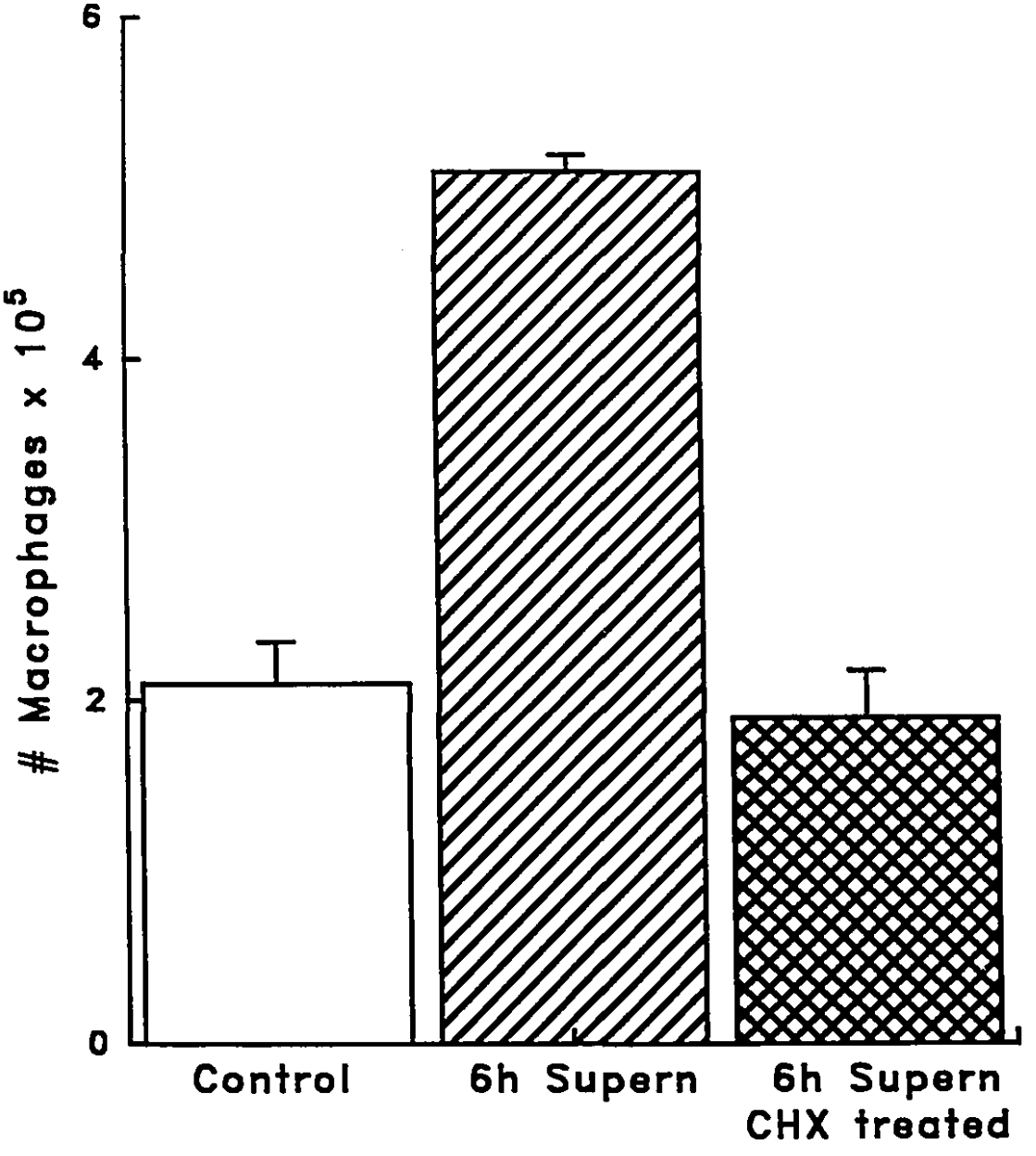


Fig. 7. Protein synthesis is required for the production of viability-sustaining activity. BMMs were pretreated with 18 mM cycloheximide for 2 h and washed four times with RPMI complete medium before infection with promastigotes. Activity of supernatants from control, infected (6 h supernatant), and CHX-pretreated infected macrophages (6 h Supern, CHX pretreated) were assayed by their ability to sustain BMMs for 72 h in the absence of exogenously added growth factor as described in Materials and Methods. Values represent the mean number of BMMs per ml (± SD) of triplicate samples. Statistical analysis was performed by Bonferroni t-test, (* significantly different from control, $p<0.05$).

DISCUSSION:

The central role of the cell-mediated immune response in the intramacrophage elimination of the *Leishmania* parasite and the resolution of visceral leishmaniasis has been well documented [1]. However, despite the considerable *in vitro* evidence for this, chronic infections are persistent in humans and mice infected with *L. donovani*, pointing to a breakdown of macrophage function and macrophage-T cell interaction in the parasite-specific immune response [2-9]. Previous studies on the mechanism of intracellular survival of *L. donovani* have focused on the negative regulation of macrophage function by this protozoan [2-9]. In the present work, we describe a novel enhancement of macrophage viability resulting from intracellular parasitization by *L. donovani*. We show that infection with *L. donovani* enhances macrophage survival in the absence of exogenous growth factor. We demonstrate that this can be attributed to the elaboration of a soluble factor into the cell culture media by infected macrophages which enhances host cell viability but not cell proliferation, and that protein synthesis is required for this process.

Cells of the haematopoietic system have brief life expectancies and their rapid turnover is thought to be controlled by a process known as programmed cell death or apoptosis [22]. Cells of the mononuclear phagocyte system circulate in the blood for 12 to 72 h, during which time they may be recruited into the tissues, where they differentiate into macrophages. The survival, differentiation, and accumulation of macrophages are

influenced by colony-stimulating factors, primarily M-CSF and GM-CSF [12,13,23]. When cultured in the absence of exogenous growth factor, macrophages undergo rapid cell death, resulting in the loss of approximately 70% of the cells within 48 h [10,11]. In this paper, we report that macrophages which are infected with promastigotes or amastigotes of *L. donovani* exhibit an increase in viability in the absence of exogenous growth factor as compared with noninfected macrophages. This pattern of response could not be induced by the uptake of latex beads by macrophages, indicating that this enhancement of cell viability is not a general phagocytosis-mediated event. An avirulent strain of *L. donovani* promastigote which is cleared by the macrophage within 24 h of inoculation likewise did not enhance macrophage viability. Thus, these results show that the increase in host cell viability was specific to *Leishmania* and required sustained intramacrophage infection by the parasite.

In an effort to characterize the enhancing effect of *Leishmania* infection on macrophage function, we examined the phagocytic capacity of control and infected macrophages after 48 h in the absence of exogenous growth factor. We demonstrate that macrophages infected with *L. donovani* exhibit increased phagocytosis of latex beads compared with control macrophages and macrophages which were grown continuously in the presence of M-CSF. In addition, macrophages infected with *L. donovani* contained more latex beads per macrophage than did control cells or cells grown continuously in M-CSF. These results argue that *L. donovani*-infected macrophages were more biologically active than control macrophages. This phenomenon could have relevance *in*

vivo, where enhanced phagocytic capacity of infected cells could lead to increased infection of macrophages by *L. donovani*.

We observed that culture supernatants from *L. donovani*-infected macrophages from 3 to 24 h after infection were able to enhance the viability of BMMs in the absence of macrophage growth factor. The viability-enhancing activity in infected cell supernatants could abrogated by pretreatment of macrophages with the protein synthesis inhibitor, cycloheximide, for 2 h prior to inoculation with *L.donovani*. To determine whether the observed enhancing activity of infected cell supernatants could be due to an increase in cell proliferation, we examined the uptake of [^{3}H]thymidine into BMMs as a measure of DNA synthesis. We demonstrated that while supernatants from macrophages infected with either the 1S2D or LV9 strain of *L. donovani* induced an increase in BMMs viability, there was no effect on DNA synthesis. These findings demonstrated that during ongoing leishmanial infection macrophages secreted a soluble factor(s) into the culture medium which acted to enhance cell viability in a manner which was shown to be independent of cell proliferation and that protein synthesis was essential for the elaboration of this factor(s).

The ability of *L. donovani* to induce the production of a soluble factor which enhances host cell viability could represent a potentially important mechanism for this protozoan infection. The possibility also remains that *L. donovani* itself elaborated this factor(s). This could facilitate the spread of infection by increasing the number of host

cells for parasitization by *L. donovani* and by increasing the number of circulating infected macrophages for uptake by the sandfly vector. The macrophage growth factor, GM-CSF is a potential cytokine which could be produced by infected macrophages to act in an autocrine fashion to enhance macrophage viability. Experimental studies on the involvement of GM-CSF in leishmaniasis have yielded conflicting results. Several studies have reported acceleration of the cure of leishmaniasis by activation of the macrophage by GM-CSF to kill the parasite [24-26]. In other instances, GM-CSF has been implicated as a disease-exacerbating cytokine in cutaneous leishmaniasis [27-28]. It was recently shown that macrophages from mice susceptible to *L. mexicana amazonensis*, the causative agent of cutaneous leishmaniasis, exhibit higher production of GM-CSF than macrophages from resistant mice [29]. As well, this cytokine has been shown to be a growth factor for *L. mexicana amazonensis* by protecting promastigotes from heat induced death [30-31]. Thus far, we have been unable to demonstrate involvement of GM-CSF in the enhancement of cell viability in our system. Neutralizing antibodies against GM-CSF were ineffective in reducing the activity of infected cell supernatants, implying that this cytokine in not the viability enhancing factor present in our supernatants (unpublished results). As well, several cytokines produced by macrophages, such as TNF-α and TGF-ß [31-36] can act synergistically with the colony-stimulating factors to enhance viability and we are continuing to investigate these possibilities. It was recently shown that TGF-ß was important for the determination of *in vivo* susceptibility to experimental cutaneous leishmanial infection with *L. amazonensis* and *L. braziliensis*. In addition, *in vitro* infection of murine peritoneal macrophages with *L. amazonensis* induced the

production of biologically active TGF-ß 72 h after infection [37].

In summary, we have described a novel positive biological effect of *L. donovani* infection on macrophage viability. Future studies must focus on identifying the molecular basis for this phenomenon. As macrophages are the only cell type which sustains *L. donovani* infection, this mechanism may play an important role in the infection process.

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CONNECTING STATEMENT TO MANUSCRIPT III

In manuscript II, a novel enhancement of macrophage function during intracellular infection with *Leishmania* was described. It was established that intramacrophage infection by *L. donovani* enhanced macrophage viability in the absence of growth factor, and that this was due, at least in part, to the elaboration of a soluble factor(s) into the culture medium by infected macrophages. This soluble factor(s) was shown to enhance macrophage viability, but not tostimulate DNA synthesis. The ensuing manuscript is a continuation of the previous study, and examines the possible mechanism by which *L. donovani* enhanced the viability of the host cell.

The observation that *L. donovani* enhanced the viability of its host cell, in a manner independent of cell proliferation, prompted an investigation of the effect of this protozoan on macrophage cell death. For haematopoietic cells, the signal to die appears to come from the environment, due to exposure to, or removal of, a specific growth factor, triggering a process of cellular self-destruction termed apoptosis. This physiological suicide mechanism is an active process, requiring specific gene transcription and protein synthesis in the targeted cell prior to death. Interference with macrophage apoptosis would represent a potential mechanism for *L. donovani* to enhance the viability of its host cell in the absence of cell proliferation. The following study examines the effect of *L. donovani* infection on macrophage apoptosis, and cytokine production.

MANUSCRIPT III

INTRACELLULAR INFECTION BY *LEISHMANIA DONOVANI* INHIBITS MACROPHAGE APOPTOSIS[1]

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FOOTNOTES:

1. Acknowledgements:

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2. Abbreviations:

LPG, lipophosphoglycan; BMM, bone marrow-derived macrophage; LCM, L929 conditioned medium; RT-PCR, reverse transcription polymerase chain reaction; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; PKC, protein kinase C; TGF-ß, transforming growth factor beta

ABSTRACT:

The phagocytic macrophage plays a critical role in host immune responses to microbial infection, and represents a major source of inflammatory and growth cytokines. Intramacrophage infection by the protozoan parasite *Leishmania donovani* results in increased viability of the host cell in the absence of exogenous growth factor. We demonstrate within that infection of bone marrow derived macrophages (BMMs) by *L. donovani* promastigotes or treatment of BMMs with lipophosphoglycan (LPG)[2], the major surface molecule of the promastigote, inhibits apoptosis in the macrophage induced by the removal of macrophage (M)-CSF. This effect was also achieved by supernatants collected from *L. donovani*-infected macrophages, implicating the elaboration of a soluble factor by infected cells as the mediator of this inhibition. To identify candidate factors, RT-PCR was employed to characterize the mRNA cytokine profile of infected macrophages. *L. donovani* infection of BMMs was found to induce gene expression for granulocyte-macrophage (GM)-CSF, TNF-α, TGF-ß and IL-6, but not M-CSF or IL-1ß. Of the cytokines induced by *L. donovani*, recombinant TNF-α and rGM-CSF were shown to inhibit apoptosis of BMMs induced by the removal of M-CSF. The amount of these cytokines in *L. donovani* infected cell supernatants was quantified by ELISA assay, and significant amounts of TNF-α, but no GM-CSF, were detected. The mechanism by which *L. donovani* may inhibit apoptosis is discussed within.

INTRODUCTION:

The macrophage is the exclusive host cell of the intracellular protozoan parasite *Leishmania*. Intramacrophage infection by *L. donovani* results in potentially fatal visceral infections in humans and the elimination of *Leishmania* parasites by the macrophage is dependent upon the mounting of an effective cell mediated immune response by the mammalian host [1]. However, this protozoan appears to be able to circumvent the specific immune response leading to macrophage activation [2-9], in part by blocking the T cell response, resulting in persistent visceral infection. The major surface molecule of *Leishmania* promastigotes, lipophosphoglycan (LPG)[2] [10], plays a key role in the interaction of *Leishmania* with the macrophage during attachment and internalization [11,12], as well as in the survival of this protozoan within this cell [13,14]. Within its mammalian host, *Leishmania* colonizes the macrophage system of the liver, spleen and bone marrow. As the bone marrow represents a natural site of infection, and the culture of these cells has been well characterized, this system is highly suited for *in vitro* study. The culture of macrophages derived from bone marrow exudates requires the presence of macrophage (M)-CSF for sustained proliferation and differentiation [15-16]. Upon removal of this growth factor *in vitro*, BMMs[3] undergo a rapid decline in cell viability resulting in the death of approximately 70% of the population within 48 h [17]. We have recently shown that BMMs infected with *L. donovani* demonstrate enhanced viability in the absence of growth factor and that this was due to the elaboration of a soluble factor(s) by infected macrophages [18]. This study examines the mechanism by which *L. donovani*

enhances the viability of its host cell.

Programmed cell death, or apoptosis, is a signal-dependent physiological suicide mechanism which preserves homeostasis by maintaining the delicate balance between cell proliferation and cell death [19]. Apoptosis is an active process of cellular self-destruction which requires specific gene transcription and protein synthesis in the targeted cell before death [20-22]. The signal to die appears to come from the environment due to exposure to or removal of, a specific growth factor or hormone [17,21-27] Although the mechanism of activation of apoptosis is different in different cell types, once triggered, the process is remarkably similar among tissues and species [28]. Cells undergoing apoptosis demonstrate nuclear condensation and degradation of the DNA into nucleosome-sized fragments by an endogenous endonuclease, followed by extensive membrane blebbing and cell fragmentation [29]. This active cell death is morphologically distinct from the passive and unregulated death by necrosis, which ultimately culminates in cell lysis.

In the present work, we demonstrate that infection of BMMs by *L. donovani* promastigotes or treatment of BMMs with LPG inhibits apoptosis of the macrophage in the absence of growth factor. Conditioned medium from infected macrophages alone was sufficient to mediate this inhibition of apoptosis of BMMs, implicating the release of a soluble factor by infected macrophages as a possible mechanism of this inhibition. In an effort to identify candidate factors we used RT-PCR to identify cytokine genes induced during infection. The mechanism(s) in which *L. donovani* inhibits apoptosis is discussed within.

MATERIALS AND METHODS:

Reagents

RPMI 1640 with L-glutamine, D-MEM with L-glutamine and HBSS without calcium and magnesium were purchased from Gibco/BRL Life Technologies (Burlington, Ont.). Low endotoxin FCS was obtained from Hyclone Laboratories Inc. (Logan, UT). Anti-murine TNF-α monoclonal antibody, and recombinant murine TNF-α (sp. activity; 2 x 10^8 U/mg) and IL-6 (5 X 10^6 U/ug) were purchased from Gibco/BRL Life Technologies. Recombinant murine GM-CSF was purchased from Genzyme Corp. (Markham, Ont.) and recombinant murine TGF-ß was purchased from R & D Systems (Minneapolis, MN).

Preparation and culture of BMMs

Six- to eight-week-old female BALB/c mice (Charles River, St. Constant, Que) were used as a source of bone marrow cells for all studies. BMMs were prepared as previously described [6-9]. Femurs were flushed with RPMI 1640 complete medium containing 10% heat-inactivated FCS, 10 mM HEPES and 100 U/ml each of penicillin and streptomycin. Bone marrow cells were incubated 18 h in tissue culture dishes (Falcon 3003) in the presence of 10% L929 cell conditioned medium (LCM) as a source of M-CSF to allow differentiated cells to adhere. Non-adherent, immature cells were collected and cultured for an additional 5 days in 10% LCM to induce macrophage differentiation and this was carried out in polystyrene culture dishes (Falcon 1029) which are weakly adherent for macrophages. The resulting macrophage population was made

quiescent as follows; 18 h before use, cells were washed in ice-cold HBSS without calcium and magnesium and detached from dishes using a cell scraper. BMMs were resuspended in RPMI complete medium in the absence of exogenous LCM. Viability was determined by trypan blue exclusion and counting live cells with a hemacytometer.

Culture and infection of BMMs with *Leishmania*

L. donovani (1S2D strain) amastigotes were isolated from the spleens of infected Syrian hamsters as previously described [30]. Promastigotes of *L. donovani* were derived by culturing amastigotes at 27°C, 5% CO_2 in modified D-MEM supplemented with 10% heat-inactivated FCS [31]. These organisms were maintained for a maximum of three passages *in vitro* and were used in the stationary phase of growth [32]. Quiescent BMMs were plated in 100mm polystyrene dishes which are weakly adherent for macrophages (Falcon 1029). Promastigotes were added to quiescent BMMs at a ratio of 7:1 for 3 h, after which time noningested promastigotes were washed away. Adherent and nonadherent BMMs were harvested in the following manner. Nonadherent cells were collected from supernatants and loosely adherent BMMs washed in ice-cold HBSS without calcium and magnesium causing detachment of loosely adherent cells which were collected using a cell scraper. In this manner, both adherent and nonadherent BMMs were counted. Cell counts were determined by trypan blue exclusion using a hemacytometer (0.1mm). Triplicate samples were taken for each group. The percentage of infected cells was routinely >95% after 3 h with four to six amastigotes per macrophage as determined by cytospin and Giemsa staining.

L. donovani infected BMM supernatants

Quiescent BMMs were infected with promastigotes at a ratio of 10:1 promastigotes per macrophage for 3 h. BMMs were washed three times to remove excess parasites and resuspended in RPMI complete medium. Giemsa staining was used to monitor the level of infection which was routinely 4-6 amastigotes/macrophage. Infected and non-infected cell supernatants were collected 6, and 24 h after inoculation, centrifuged at 100*g* and filtered before use. Supernatants were assayed at 75% strength.

Treatment of BMMs with infected cell supernatants and recombinant cytokines

BMMs were made quiescent by the removal of LCM conditioned medium for 18 h before use. Quiescent cells were then treated for 24 h with either *L. donovani*-infected cell supernatant, 20% LCM conditioned medium as a source of M-CSF, or one of the following recombinant murine cytokines: rTNF-α (1 ng/ml), rGM-CSF (100 ng/ml), rTGF-ß (1 ng/ml) or rIL-6 (10 ug/ml). In neutralization studies, promastigote-infected macrophages were incubated with rat anti-murine TNF-α monoclonal antibody (15 ug/ml) for 24h prior to the extraction of fragmented DNA. Media containing rTNF-α and anti-TNF-α antibody were preincubated for 2 h prior to addition to BMMs for 24 h.

Treatment of BMMs with LPG

The LPG used in this study was derived from *L. donovani* promastigotes of the 1S2D strain and was kindly donated by Dr. S.J. Turco. Before use, the LPG was resuspended in RPMI complete medium and sonicated for 10 s. BMMs were made

quiescent by the removal of LCM conditioned medium for 18 h before the addition of LPG. To determine the effect of LPG on macrophage apoptosis, BMMs were treated with 0.5, 1, 3, and 7 uM LPG for 24 h. Apoptosis was determined by extraction of fragmented DNA and resolution by agarose gel electrophoresis. To examine the effect of LPG on cell viability, quiescent BMMs were treated for 48 h with 7 uM LPG. As a comparison, quiescent BMMs were also treated with 20% LCM as a source of M-CSF, or infected with *L. donovani* promastigotes for 48 h. Viability was determined by trypan blue exclusion and counting live cells with a hemacytometer.

DNA fragmentation assay

Internucleosomal degradation of macrophage DNA was used as an indication of apoptosis. DNA fragmentation was assessed by the isolation of oligonucleosome-length DNA. Because recognition and ingestion of dead cells by macrophages is very rapid [33], the proportion of laddered chromatin at any one time is quite small relative to intact chromatin [34]. Because this large excess of intact chromosomal DNA can obscure any laddered DNA present, we have applied a protocol which isolates only fragmented DNA and the experiments were normalized by using equal numbers of macrophages from each test group as previously described [34]. In brief, 6 x 10^6 BMMs from each treatment were washed in PBS and lysed in 10 mM Tris pH 7.4, 5 mM EDTA, 1% Triton X-100 for 20 min on ice. After centrifugation at 11,000*g* for 20 min, supernatants containing fragmented DNA were removed and digested with 20 ug/ml RNAse A at 37°C for 1 h and 0.1 mg/ml Proteinase K for an additional hour. The DNA was extracted using

phenol-chloroform, precipitated in ethanol and centrifuged for 20 min at 11,000*g*. The DNA was dissolved in 30 ul TE pH 8.0 and the entire sample was electrophoresed on a 1.2% agarose gel for 1 h at 85v.

RNA amplification by PCR

Total cellular RNA was isolated from 5 x 10^6 BMMs using RNAzol B RNA isolation reagent according to the manufacturer's instruction (Biotex International Inc., Friedswood, TX). Complementary DNA was prepared from total RNA using a reverse transcription system (Promega Corporation, Madison, WI). Briefly, 1 μg of RNA was added to 15 U of AMV reverse transcriptase, 1mM each of dNTP, 0.5 μg of oligo$(dT)_{15}$ primer and 5 mM $MgCl_2$ in a total volume of 40 μL. The reaction was incubated at 42°C for 1 h followed by 5 min at 99°C to inactivate the enzyme. Four μL of each cDNA was subjected to PCR amplification using the GeneAmp kit (Perkin-Elmer Cetus Co., Norwalk, CT) in a 50 μL reaction containing 1.25 U of Amplitaq DNA polymerase, 200 μM of each dNTP, and 1 μM of the appropriate cytokine primer (Stratagene Cloning Systems, La Jolla, CA). The PCR reaction conditions consisted of a 5 min denaturation at 94°C, a 5 min annealing at 60°C, followed by 35 cycles of 1.5 min at 72°C, 45 sec. at 94°C and 45 Sec. at 60°C, with a final extension of 10 min at 72°C. An aliquot of the PCR reaction was electrophoresed through a 1.0% agarose gel containing 0.5 μg/mL ethidium bromide.

TNF-α and GM-CSF ELISA

An ELISA assay (Cedarlane Laboratories, Hornby, Ont.) was used to quantify the amount of TNF-α and GM-CSF in control and *L. donovani*-infected cell supernatants. These ELISAs were specific for the measurement of TNF-α or GM-CSF and did not cross-react with other cytokines. All samples were assayed in duplicate and the absorbance of each well was read on a Bio-Tek Instruments plate reader at 490nm. Recombinant TNF-α/GM-CSF provided by the manufacturer served as standards and the positive control. The amount of TNF-α/GM-CSF in each sample was determined by interpolation from a standard curve. The minimal detectable level of the assay was 5 pg/ml for GM-CSF and 25 pg/ml for TNF-α.

RESULTS:

***Leishmania donovani* infection inhibits macrophage apoptosis**

It has previously been reported that monocyte apoptosis can be inhibited by the colony-stimulating factors [16-17]. Because BMMs were used in our studies with *L. donovani*, we first examined apoptosis in BMMs in the presence and absence of M-CSF (LCM as the source of M-CSF). We routinely culture BMMs in 10% LCM for 5 days to allow cell maturation and differentiation. As shown in Figure 1, there was apoptotic DNA fragmentation in 5-day-old BMMs grown either continuously in 10% M-CSF *(lane 1)* and 5-day-old BMMs deprived of M-CSF for 24 h but maintained in RPMI containing 10% FCS *(lane 3)*. These results demonstrated that although 10% LCM (M-CSF) maintained the proliferation and differentiation of macrophages derived from the bone marrow, a certain percentage of this cell population, after being cultured for 5 days in 10% LCM, was undergoing apoptosis. Based upon this finding, 5-day-old BMMs were supplemented with fresh 20% LCM to inhibit apoptosis in further experiments. Serum starvation is known to induce apoptosis in a variety of cell types, and in accordance with this, removal of serum and M-CSF from 5-day-old BMMs for 24 h resulted in cells undergoing apoptosis *(lane 2)*. In direct contrast, apoptosis in 5-day-old BMMs induced by the removal of M-CSF was prevented by infection with *L. donovani* promastigotes for 24 h before extracting DNA *(lane 4)*. Similar results were obtained when this experiment was performed in the presence of 3 ug/ml polymixin B, demonstrating that this inhibition of apoptosis was not due to any contamination by endotoxin. The inhibition of apoptosis

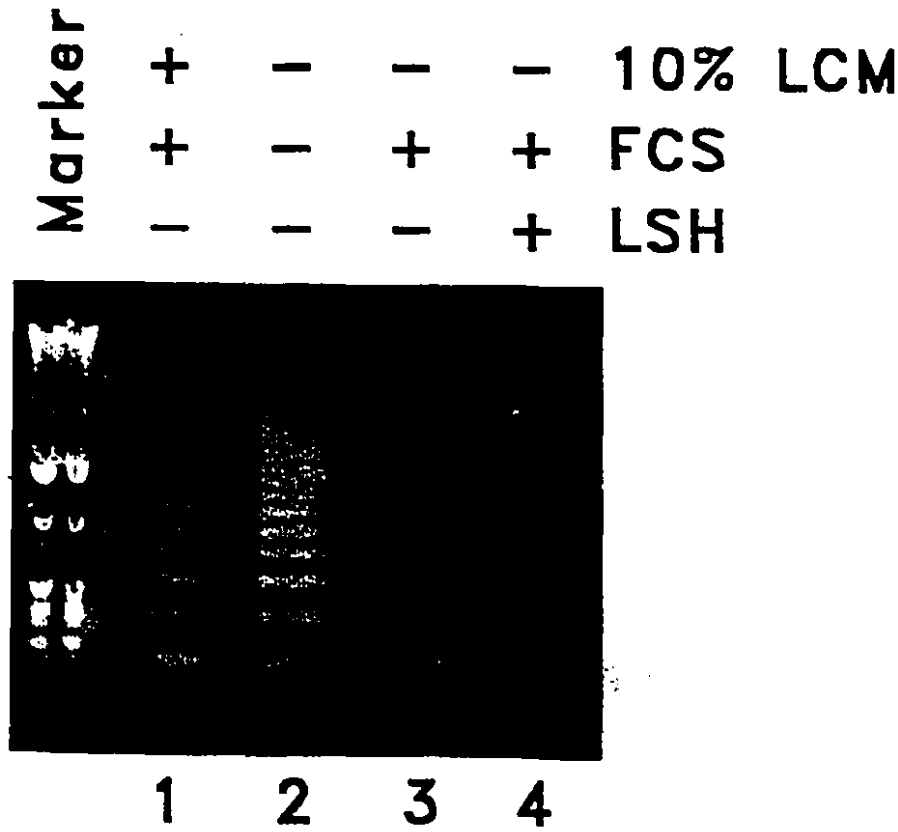

Figure 1. ***Leishmania donovani*** **infection inhibits apoptosis.** Electrophoretic pattern of nucleosomal DNA fragments isolated from BMMs. Low molecular weight DNA from cells was isolated as described in Materials and Methods from an equal number of BMMs after the following treatments; *lane 1*, 5-day-old BMMs cultured in media containing FCS and 10% LCM as a source of M-CSF; *lane 2*, BMMs starved of FCS and M-CSF for 24 h; *lane 3*, BMMs cultured in media containing FCS but in the absence of M-CSF for 24 h; *lane 4*, BMMs infected with *L. donovani* promastigotes for 24 h, cultured in media containing FCS, but no M-CSF. Molecular weight markers are 1 kb DNA ladder from Gibco. Results are representative of three experiments. Note, lane 1 represents cells grown continuously in 10% LCM for 5 days.

in infected macrophages is consistent with our previous work which demonstrated that *L. donovani* infection enhanced macrophage viability in the absence of growth factor [18].

BMMs infected with *L. donovani* promastigotes secrete a soluble factor that inhibits macrophage apoptosis

In order to determine if the inhibition of apoptosis induced by *L. donovani* infection could be due to the elaboration of a soluble factor by infected macrophages, we examined apoptosis in BMMs exposed to supernatants derived from infected cells. BMMs were treated for 24 h with either control supernatant collected from BMMs deprived of M-CSF for 6 and 24 h, or, supernatant collected from BMMs infected with *L. donovani* promastigotes for 6 and 24 h. As shown in Figure 2, while apoptosis occurred in BMMs cultured in control supernatants *(lanes 1 & 3)*, no such evidence of apoptosis was found in BMMs cultured in infected cell supernatants collected 6 *(lane 2)* and 24 h *(lane 4)* post-infection. These findings imply that a soluble factor was secreted by *L. donovani* infected macrophages into the culture medium which may act in an autocrine manner to prevent cell death by apoptosis. These data are consistent with our previous observations that supernatants from *L. donovani* infected macrophages could enhance viability of BMMs cultured in the absence of growth factor [18].

***L. donovani* infection of BMMs induces cytokine gene expression**

A possible explanation for the above observations was that *L. donovani*-infected BMMs secrete cytokine(s) which may act in an autocrine manner to prevent macrophage

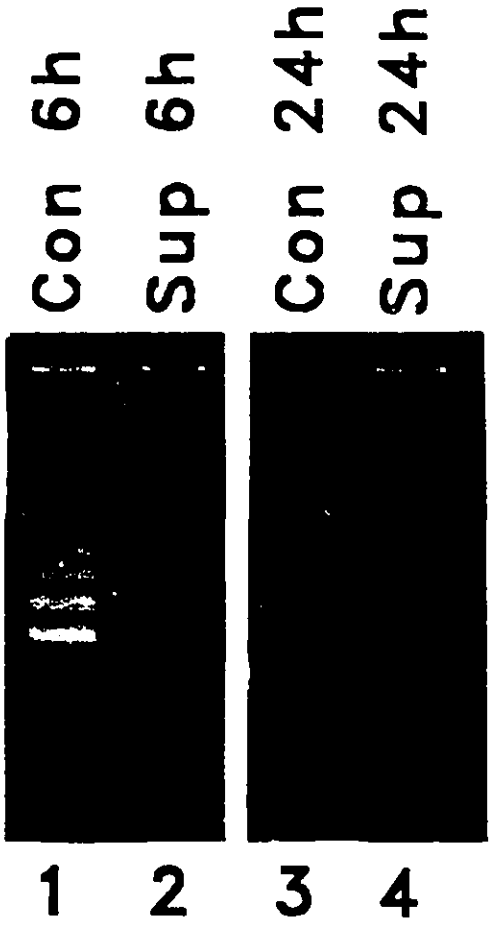

Figure 2. ***Leishmania donovani*-infected cell supernatant inhibits apoptosis.** Electrophoretic pattern of nucleosomal DNA fragments isolated from BMMs. Low molecular weight DNA was extracted from an equal number of BMMs after 24 h in the following media; *lane 1*, supernatant that was in contact with control noninfected macrophages for 6 h; *lane 2*, supernatant that was in contact for 6 h with *L. donovani*-infected macrophages; *lane 3*, supernatant that was in contact for 24 h with control noninfected macrophages; *lane 4*, supernatant that was in contact for 24 h with *L. donovani*-infected macrophages. Results are representative of three experiments.

apoptosis. In an effort to investigate this possibility, RT-PCR was employed to characterize the cytokine gene expression profile of BMMs infected with *L. donovani*. Total cellular RNA was extracted from BMMs 3, 6, 9 and 24 h postinoculation with *L. donovani* promastigotes and reverse transcribed to cDNA which was used as a template for PCR. As a control for non-infected cells, the same procedure was carried out on quiescent BMMs cultured in the absence of M-CSF for 18 h. Actin mRNA was used as a control for this analysis and confirmed that RNA isolated from infected and non-infected BMMs was intact. As shown in Figure 3B, infection of BMMs by *L. donovani* promastigotes induced the expression of four cytokine genes: GM-CSF, TNF-α, TGF-ß and IL-6. Expression of the GM-CSF, TNF-α and IL-6 genes was detected as early as 3 h postinoculation, which represented the first possible time point as this was the time required for infection. Expression of the TGF-ß gene was induced later than the other cytokines, appearing only 9 h postinfection. The expression of all four cytokine genes was still apparent 24 h postinfection. *L. donovani* infection of BMMs did not stimulate the expression of the M-CSF gene *(Fig. 3A)*. In this case, the positive control was RNA isolated from L929 cells. IL-1ß mRNA was present in both quiescent and *L. donovani* infected BMMs *(Fig. 3A)*.

Effect of recombinant cytokines on BMMs apoptosis

The RT-PCR analysis revealed that a number of cytokine genes were expressed in *L. donovani*-infected cells. We were interested in determining whether any of these cytokine gene products could inhibit apoptosis in the BMMs used in this study.

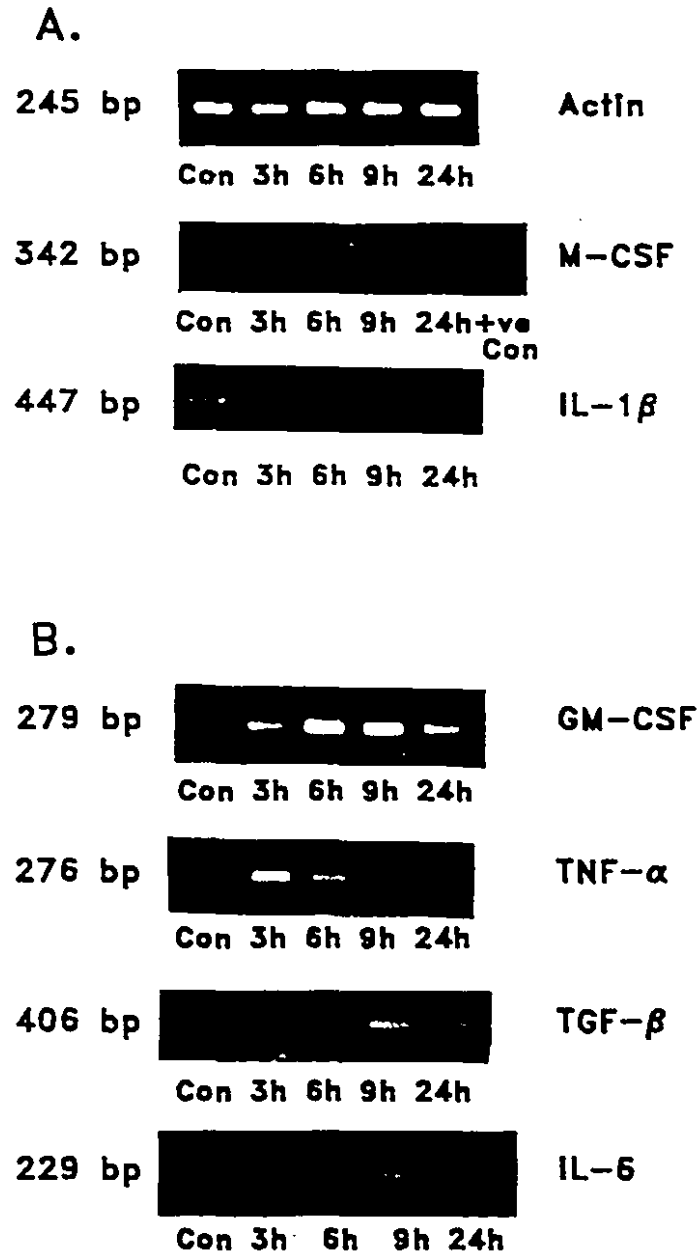

Figure 3. Effect of *L. donovani* infection on cytokine gene expression in BMMs. RT-PCR analysis of cytokine mRNA in BMMs during infection with *L. donovani* was performed as described in Materials & Methods. One μg of total RNA from control uninfected BMMs *(lanes labelled Con)* or macrophages infected for 3, 6, 9, or 24 h with *L. donovani* promastigotes *(lanes labelled 3h, 6h, 9h, 24h)* was reverse transcribed to cDNA. The cDNA sample was used as a template for 30 cycles of PCR, and the products were electrophoresed through a 1% agarose gel containing ethidium bromide. (A) Cytokine gene expression unaltered by *L. donovani* infection. L929 mRNA was used as a positive control for M-CSF. (B) Cytokine gene expression induced by *L. donovani* infection. Each result is representative of at least three experiments.

Quiescent BMMs were treated with recombinant TNF-α, rGM-CSF, rTGF-ß or rIL-6 for 24 h prior to harvesting of fragmented DNA *(Fig. 4)*. The controls *(Fig. 4A & B - lane 1)* were quiescent BMMs cultured for 24 h in media containing no M-CSF. Treatment of BMMs with *L. donovani* infected cell supernatant *(4A, lane 2)* and 20% LCM as a source of fresh M-CSF *(4A, lane 3)* served as controls for the abrogation of BMM apoptosis. Both rTNF-α *(4A, lane 4)* and rGM-CSF *(4A, lane 5)* were capable of inhibiting apoptosis, as demonstrated by the reduced level of fragmented DNA in these treated BMMs. Treatment with either rTGF-ß or rIL-6 did not alter the level of macrophage apoptosis, as the characteristic DNA fragmentation occurred in rTGF-ß *(4A, lane 6)* and rIL-6 *(4B, lane 2)* treated BMMs cell lysates. These data are consistent with several reports showing that GM-CSF and TNF-α prevent apoptosis in monocytes [17,23], and that TGF-ß and IL-6 do not inhibit monocyte apoptosis [17,23]. As demonstrated in this experiment, supernatants from *L. donovani*-infected cells was as effective at preventing apoptosis in these quiescent BMMs as recombinant TNF-α, rGM-CSF, or 20% LCM.

Quantitation of TNF-α and GM-CSF in *L. donovani*-infected cell supernatants

The RT-PCR analysis revealed that *L. donovani*-infected BMMs expressed the TNF-α and GM-CSF genes, and the data presented in Figure 4 confirmed that both TNF-α and GM-CSF could impair apoptosis in the BMMs used in this study. We were therefore interested to determine whether *L. donovani*-infected BMMs secreted TNF-α and GM-CSF into the culture media. The amount of TNF-α and GM-CSF in *L.*

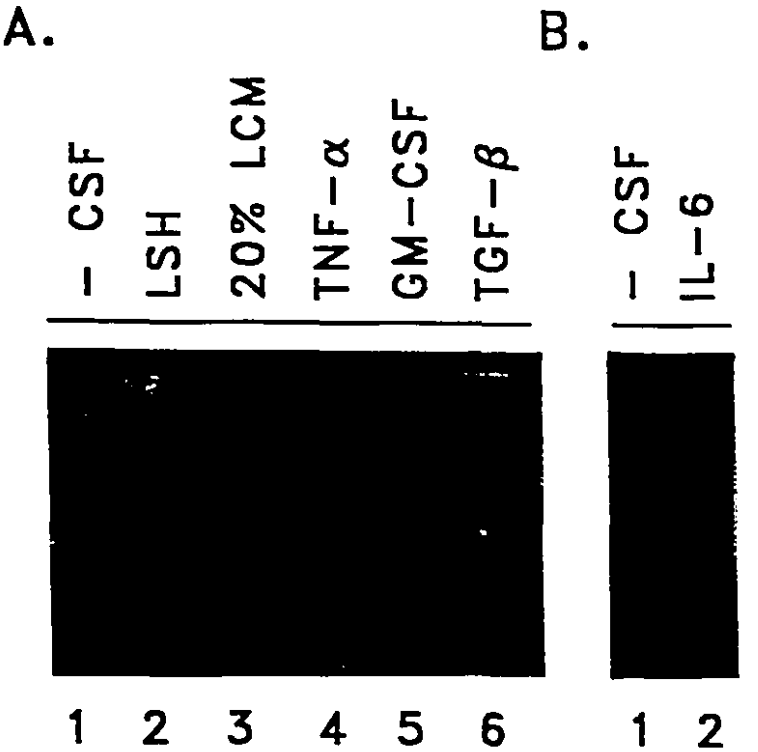

Figure 4. Effect of recombinant cytokines on apoptosis of BMMs. Comparison of electrophoretic pattern of nucleosomal DNA fragments isolated from BMMs after treatment with recombinant cytokines or *L. donovani*-infected cell supernatant. Low molecular weight DNA from cells was isolated as described in Materials and Methods from an equal number of macrophages after treatments; (A) ***lane 1,*** BMMs cultured in the absence of M-CSF for 24 h; ***lane 2,*** BMMs treated with cell supernatant from macrophages infected with *L. donovani* promastigotes for 24 h; ***lane 3,*** BMMs treated with fresh 20% LCM conditioned medium as a source of M-CSF for 24 h; ***lane 4,*** BMMs treated with 1 ng/ml of rTNF-α for 24 h; ***lane 5,*** BMMs treated with 100 ng/ml of rGM-CSF for 24 h; ***lane 6,*** BMMs treated with 1 ng/ml of rTGF-ß for 24 h; **(B)** ***lane 1,*** BMMs cultured in the absence of M-CSF for 24 h; ***lane 2,*** BMMs treated with 10 ng/ml of rIL-6 for 24 h. Results are representative of three experiments.

donovani-infected (6 and 24 h) cell supernatants was determined by ELISA. Recombinant TNF-α and rGM-CSF served as positive controls and internal standards for quantification. As demonstrated in Table I, whereas a significant level of TNF-α was present in infected cell culture supernatants, no GM-CSF was detected. The highest level of TNF-α was detected in supernatants collected 6 h postinfection (1902 ± 763 pg/ml) and this had decreased approximately 10-fold by 24 h postinfection (170 ± 63 pg/ml). No detectable TNF-α or GM-CSF was found in supernatants collected from uninfected BMMs or in the L929 cell-conditioned medium which was used as a source of M-CSF for BMM culture. It is interesting that although *L. donovani*-infected BMMs express the GM-CSF gene, we were unable to detect GM-CSF protein in supernatants from infected cells. This could indicate that there is a translational control or that GM-CSF was cell surface associated. Nevertheless, cell surface associated GM-CSF would not have been responsible for the apoptosis inhibiting activity observed in the supernatants from *L. donovani*-infected cells.

The level of TNF-α released 6 h following infection (1.9 ng/ml) was more than the amount (1.0 ng/ml) of rTNF-α used to prevent apoptosis in quiescent BMMs (data from Fig. 4). These data suggest that the release of TNF-α from infected cells may be capable of acting in an autocrine manner to inhibit apoptosis. To determine if inhibition of apoptosis by *L. donovani* infection was due to the release of TNF-α from infected cells, BMMs infected with *L. donovani* promastigotes were treated with neutralizing anti-TNF-α antibody (Fig. 5A). Anti-TNF-α antibody failed to restore the ability of infected BMMs to undergo apoptosis *(lane 4)*, indicating that the release of TNF-α from infected

TABLE 1:

Levels of TNF-α and GM-CSF induced by *L. donovani* infection of BMMs.

Supernatant	amount of TNF-α[a] (pg/ml)	amount of GM-CSF[a] (pg/ml)
Control[b]	0.0	0.0
L. donovani 6h infection	1902 ± 763	0.0
L. donovani 24h infection	170 ± 63	0.0
L929 conditioned medium (LCM)	0.0	0.0

[a] data is the average of three separate experiments (± standard deviation)
[b] represents supernatant from uninfected BMMs
[c] recombinant cytokines provided by the manufacturer served as positive controls which were introduced into the same media used to maintain BMMs.

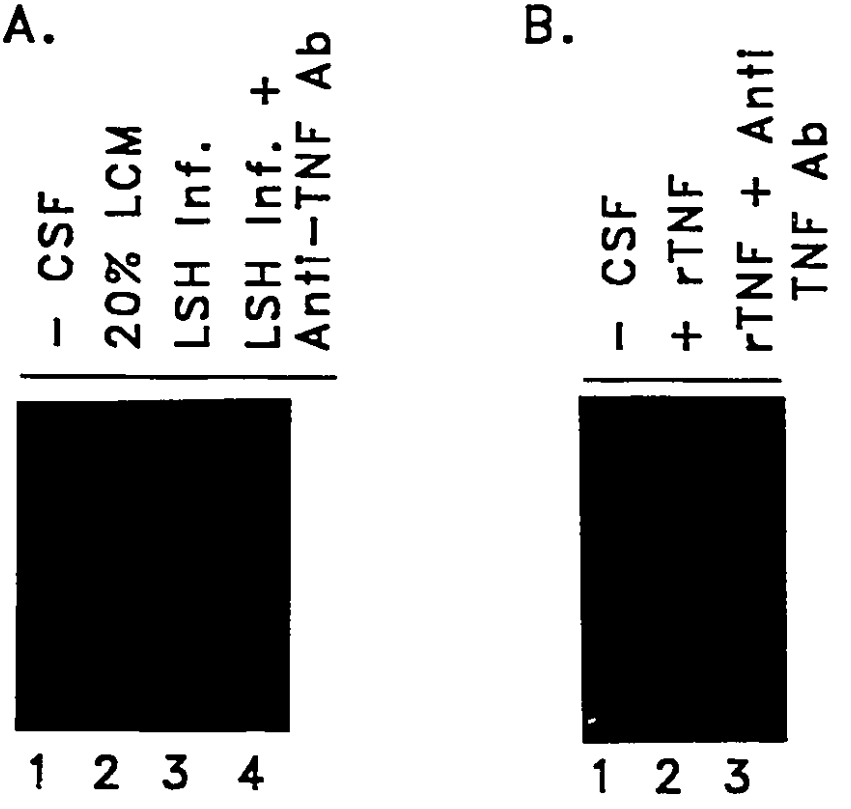

Figure 5. Effect of anti-TNF-α antibody on *L. donovani*-induced inhibition of apoptosis. Comparison of electrophoretic pattern of nucleosomal DNA fragments isolated from BMMs. Low molecular weight DNA was isolated as described in Materials and Methods from an equal number of macrophages after the following treatments; **(A)** ***lane 1,*** BMMs cultured in the absence of M-CSF for 24 h; ***lane 2,*** BMMs treated with 20% LCM as a source of M-CSF for 24 h; ***lane 3,*** BMMs infected with *L. donovani* promastigotes for 24 h; ***lane 4,*** BMMs infected with *L. donovani* promastigotes and treated with 15 ug/ml anti-TNF-α neutralizing antibody for 24 h. **(B)** ***lane 1,*** BMMs cultured in the absence of M-CSF for 24 h; ***lane 2,*** BMMs treated with 1 ng/ml rTNF-α for 24 h; ***lane 3,*** BMMs treated with 1 ng/ml rTNF-α and 10 ug/ml anti-TNF-α neutralizing antibody. Results are representative of two separate experiments.

macrophages was not solely responsible for the inhibition of apoptosis. As expected, the characteristic DNA ladder of cells undergoing apoptosis was inhibited by treatment of BMMs with either 20% LCM *(lane 2)*, or infection with *L. donovani (lane 3)*. As a control to verify that this anti-TNF-α antibody could reverse the inhibition of apoptosis by TNF-α, BMMs were treated with either rTNF-α alone or rTNF-α and anti-TNF antibody. As shown in Figure 5B, treatment with rTNF-α inhibited macrophage apoptosis as demonstrated by the absence of fragmented DNA *(lane 2)* and this abrogation of apoptosis was successfully reversed by treatment with neutralizing TNF-α antibody *(lane 3)*.

Treatment of BMMs with LPG enhances cell viability, and inhibits macrophage apoptosis

We have previously shown that *L. donovani* infection of BMMs enhances macrophage viability in the absence of growth factor [18], and the data presented herein support the idea that this could be the result of inhibition of macrophage apoptosis by the parasite. It was also of interest to investigate candidate parasite molecules which may be involved in the inhibition of apoptosis. We have chosen to examine LPG, the most abundant promastigote surface molecule. The effect of LPG on macrophage apoptosis was of particular interest, as this parasite molecule is known to inhibit protein kinase C (PKC)-mediated signal transduction [8,13,35], which has been implicated as one of the mediators of apoptosis [36,37]. As shown in Figure 6B, treatment of BMMs cultured in the absence of M-CSF with 0.5 to 7 uM LPG resulted in a dose-dependent inhibition of

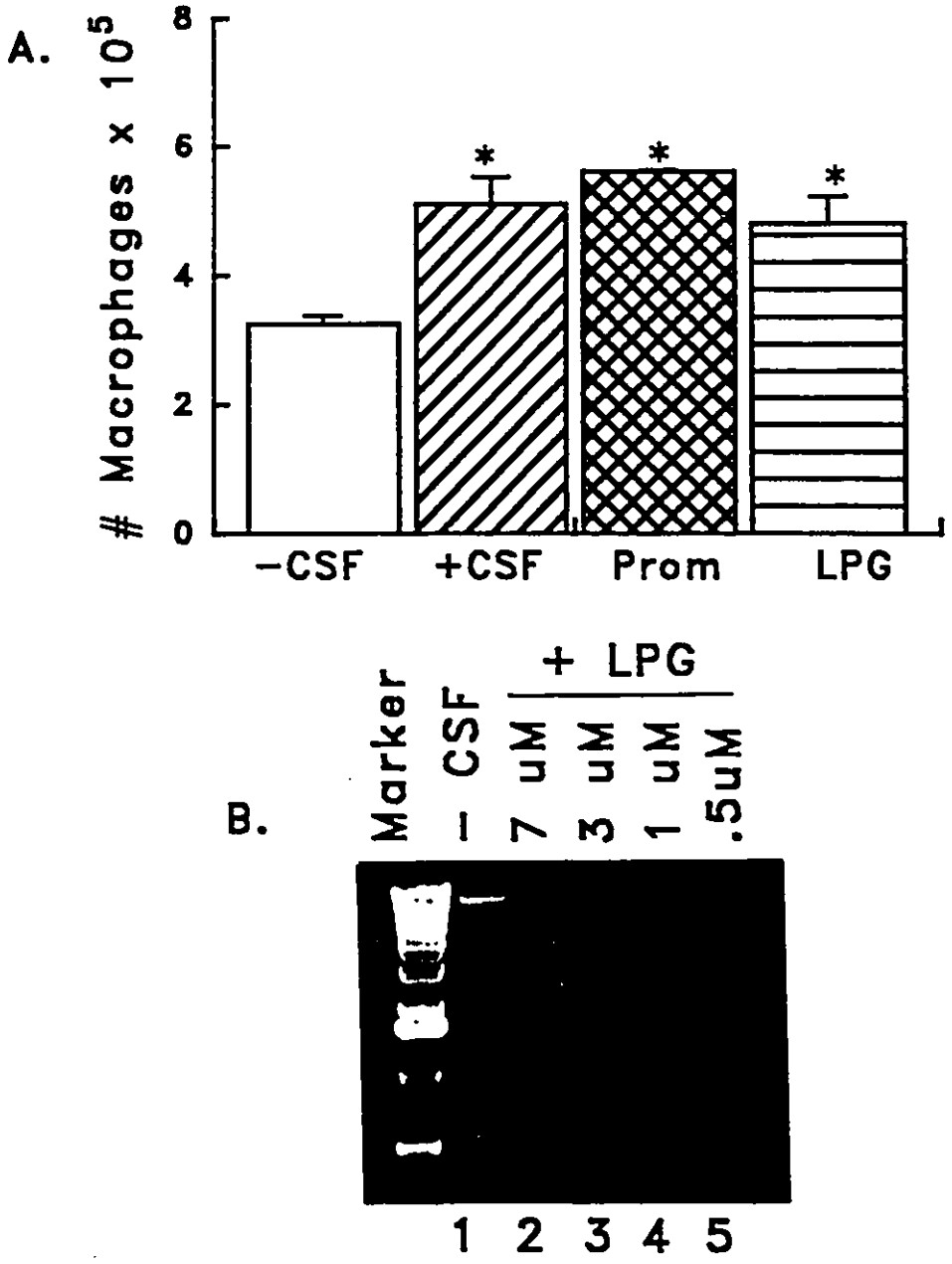

Figure 6. Viability of BMMs is enhanced by treatment with LPG. (A) BMMs were harvested and counted 48 h after the following treatments; *-CSF*, BMMs cultured in RPMI in the absence of M-CSF; *+CSF*, BMMs cultured in RPMI containing 10% LCM as a source of M-CSF; ***Prom***, BMMs cultured in the absence of M-CSF and infected with *L. donovani* promastigotes; ***LPG***, BMMs cultured in the absence of M-CSF and treated with 7 uM LPG. Values represent the mean number of BMMs/ml (± SD) of triplicate samples. Statistical analysis was performed by the Bonferroni t-test (* significantly different from control, $p<0.05$). **(B) Dose-response effect of LPG on BMM apoptosis.** Low molecular weight DNA was isolated from an equal number of BMMs after 24 h following removal of M-CSF from the media *(lane 1)* or after 24 h in the presence of differing concentrations of LPG in media devoid of M-CSF *(lanes 2-5)*. These data are representative of three different experiments.

macrophage apoptosis. Treatment of BMMs with 7 and 3 uM LPG resulted in complete abrogation of DNA fragmentation, while lower doses of LPG were less efficient at inhibiting apoptotic cell death. To determine if the abrogation of macrophage apoptosis by LPG resulted in enhanced the viability of BMMs quiescent BMMs were treated with 7 uM LPG for 48 h. As shown in Figure 6A, LPG treatment of BMMs resulted in an enhancement of cell viability comparable to that obtained with infection of BMMs with *L. donovani* promastigotes *(Prom)* or treatment with 10% LCM containing M-CSF for 48 h. These data suggest that the enhancement of BMM viability and the inhibition of macrophage apoptosis mediated by *L. donovani* in the absence of growth factor, could be attributed in part to the promastigote molecule LPG.

DISCUSSION:

The major observation reported in the present study is that *L. donovani* infection or LPG, the major surface molecule of *Leishmania* promastigotes, inhibited apoptosis in BMMs. This is consistent with our previous observation that in the absence of M-CSF, *L. donovani* infected BMMs survive longer and are more viable than uninfected BMMs [18]. We have also demonstrated within that *L. donovani* infection induced the expression of a number of cytokine genes. It is likely that the *L. donovani* mediated inhibition of apoptosis may be related at least in part to the induction of cellular cytokine gene expression.

We have observed that the TNF-α, GM-CSF, TGF-ß and IL-6 genes are induced in infected cells. Both infected and non-infected cells contained mRNA for actin and IL-1ß. Of the cytokines capable of inhibiting apoptosis in BMMs, we were able to detect the release of TNF-α from *L. donovani*-infected cells and this was consistent with the previous observation that *L. major*-infected macrophages also release TNF-α [38]. However, it should be noted that TNF-α production by human monocytes infected with *L. donovani* has not been observed [39]. Although TNF-α may have acted in an autocrine manner to inhibit apoptosis in infected cells, additional factors were also involved since we were unable to restore the ability of infected BMMs to undergo apoptosis with neutralizing anti-TNF-α antibodies.

We have also observed that *L. donovani* induced the expression of TGF-ß mRNA in BMMs. This is of particular interest because it has been recently shown that TGF-ß

was involved in the susceptibility to experimental cutaneous leishmanial infection with *L. amazonensis* and *L. braziliensis* [40], and that TGF-ß could suppress intracellular destruction of *L. major* [41]. The present study shows that *L. donovani*, like *L. amazonensis*, induces TGF-ß gene expression in macrophages and this may have implications for the outcome of *L. donovani* infection *in vivo*.

Macrophages represent a major source of inflammatory and growth cytokines and *L. donovani*-infected cells expressed a number of the cytokine genes examined in this study. It is likely that additional cellular genes other than those which have been identified in this study are expressed in the infected cells. The products of *L. donovani*-induced genes may act intracellularly as well as extracellularly to collectively inhibit apoptosis in infected cells. Furthermore, in addition to the induction of gene expression, *L. donovani* may impair gene expression in such a manner to inhibit apoptosis. For example, it has been demonstrated that both *L. donovani* infection and treatment of BMMs with LPG, result in an inhibition of macrophage PKC-mediated c-*fos* gene expression [7,8]. Because there is evidence that both the PKC signal transduction pathway and c-*fos* gene expression in particular are important in mediating apoptosis [36,37,42], then impairment of PKC and c-*fos* expression may act synergistically with any autocrine mechanism in preventing apoptosis. This is consistent with the present study, which revealed that LPG could impair apoptosis in BMMs. In this event, it was not surprising that the addition of neutralizing antibodies to any one cytokine, such as TNF-α, did not restore the ability of infected BMMs to undergo apoptosis. These data also suggest that the enhancement of macrophage viability by *L. donovani* could be

attributed in part to the promastigote molecule LPG. However, the observed effects of LPG on macrophages function would only be of relevance during the initial stages of infection of the macrophage by promastigotes, as amastigotes of *L. donovani* have been shown to lack LPG on their surface.

In conclusion, two major observations are reported in the present study which we believe are related. The first is that *L. donovani* infection prevented apoptosis in BMMs and that this could also be mediated in part by LPG. Second, infected BMMs express a number of cytokine genes, which probably contributes to the prevention of apoptosis and may also be important in other aspects of the infection process. The enhancement of host cell viability could facilitate the spread of infection by increasing the number of host cells for parasitization by *L. donovani* and by increasing the number of circulating infected macrophages for uptake by the sandfly vector.

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SECTION C

GENERAL DISCUSSION

DISCUSSION

Resolution of leishmanial infection requires the development of an effectual cell-mediated immune response, culminating in the activation of macrophages to a microbicidal state [1]. However, despite the substantial *in vitro* evidence for this, *L. donovani* causes persistent, and potentially fatal visceral infections in humans. Given the role of macrophages in both the initiation and the resolution of infection, the survival of this parasite within these cells must be assisted by factors that inhibit or diminish the impact of the macrophage microbicidal mechanisms. On the other hand, it may also be advantageous for *Leishmania* to enhance the viability of its host cell. While previous studies on the intracellular survival of *Leishmania* have focused primarily on the impairment of macrophage function by this protozoan [2-9], it has not been established whether *L. donovani* enhances its host cell in any way. Since the success of parasitic infection depends on the ability of an organism to survive within a host without causing its mortality in the short term, a balance must be achieved between the inhibition of host defences, and the maintainance of host survival and viability. Thus, a complete study of the effect of *Leishmania* on macrophage function must include the examination of the mechanisms by which this parasite ensures macrophage survival, while impairing such critical host cell functions. In this thesis, I have attempted to provide a more global understanding of how *Leishmania* affects the physiology of its host cell, by examining both positive and negative influences of this protozoan on the macrophage.

Manuscript I delineates the selective impairment of signal transduction by *L. donovani*, and the biological significance of this inhibition. Targeting a signal transduction pathway in the macrophage would allow this parasite to manipulate the passage of information from the cell surface to the nucleus. The two signal transduction pathways selected for study, PKC and PKA, appear to have opposing functions within the cell. The PKC signal transduction pathway mediates the signals for activation of the macrophage [10], while the PKA pathway has been shown to mediate downregulation of the immune response [11]. C-*fos* gene expression, which is differentially expressed via the PKC and PKA pathways in the macrophage [12], was selected as a nuclear marker for the study of signal transduction in *Leishmania*-infected macrophages. The c-*fos* proto-oncogene is among the 'immediate early genes' which are rapidly induced following stimulation with a wide variety of mitogens and growth factors. Its product complexes with another proto-oncogene product, Jun, to form the AP1 transcriptional activator [13]. Thus, alteration of c-*fos* gene expression would allow the modulation of multiple genes, by affecting AP1-enhanced transcription.

Considering the proposed role of PKC in macrophage activation, manipulation of cellular gene expression by *Leishmania* through PKC may aid in subverting this critical aspect of the cell-mediated immune response shown to be required for parasite killing. Previous studies from our laboratory revealed that infection of macrophages with *L. donovani* amastigotes resulted in impairment of PKC-mediated c-*fos* gene expression [6], and that the LPG molecule also impaired the PKC-, but not the PKA-associated signal transduction pathway [7,8]. Similarly, Olivier et al. revealed that *L. donovani* infection

impaired macrophage PKC function [9]. Based on these observations, it was important to determine whether *L. donovani* infection had any effect on the PKA pathway under conditions where the PKC pathway was impaired, and then to determine if impairment of signal transduction had relevance to the level of infection.

In manuscript I, the effect of *L. donovani* infection on both the PKC and PKA pathways was examined. The previous observation that infection with amastigotes of *L. donovani* impaired PKC-mediated signal transduction in the macrophage was extended to include infection with promastigotes, and also to 1 h contact of the macrophage with both promastigotes and amastigotes, in the absence of subsequent infection. Impairment of PKC-mediated gene expression by *Leishmania* was shown to require direct interaction with the macrophage, as treatment of cells with *L. donovani* conditioned medium could not emulate this effect. Together, these studies implied that the impairment of PKC by *Leishmania* was induced very soon after macrophage contact with either promastigotes or amastigotes, suggesting a potential role for this abrogation of signal transduction in the establishment of infection. Indeed we demonstrated that abrogation of macrophage PKC signal transduction, by both specific and general inhibitors, resulted in increased parasite uptake and heavier parasite loads throughout our study. Thus, these studies revealed that the impairment of PKC-mediated signal transduction by *Leishmania* had biological relevance, as simulation of this effect *in vitro* provided *Leishmania* with a significant advantage over the macrophage.

It was shown previously that *L. donovani*-infected macrophages produced increased levels of cyclooxygenase products, in particular PGE_2 [14], which had the

potential to act in an autocrine manner to stimulate the PKA signal transduction pathway. This observation motivated our investigation of the effect of *L. donovani* on PKA-mediated signal transduction in the macrophage. Earlier studies from our laboratory demonstrated that the *Leishmania* surface molecule, LPG, impaired PKC-, but not PKA-mediated c-*fos* gene expression [7]. However, the finding that this molecule was absent on amastigotes of *L. donovani* [15], prompted a more extensive investigation of the effect of *Leishmania* on PKA signal transduction. We undertook to examine the effect of live parasites, both amastigotes and promastigotes, on PKA-mediated c-*fos* gene expression, in both infected macrophages and macrophages put in contact with the parasite for 1 h. Results from these studies revealed that PKA-mediated c-*fos* expression was unaltered under conditions showing an impairment of PKC-mediated c-*fos* gene expression by *Leishmania*. Thus, although *L. donovani*-infected macrophages secreted increased levels of PGE_2 which could potentially act in an autocrine manner to stimulate the PKA pathway, we found that PKA-mediated gene expression was unaltered in infected macrophages. Consistent with this observation, it has recently been demonstrated that in spite of increased cyclooxygenase output, intracellular cAMP levels in *L. donovani*-infected macrophages were not significantly different from uninfected cells [16].

The finding that PKA-mediated c-*fos* gene expression was unaltered, under conditions showing an impairment of PKC-mediated c-*fos* expression, implied that *Leishmania* did not act as a general kinase inhibitor, but rather, selectively impaired signal transduction within the macrophage. If indeed the PKA pathway plays a role in the downmodulation of the immune response, it follows that this pathway would not be

targeted for impairment by *Leishmania*. This is consistent with the data presented in manuscript I, demonstrating that impairment of both the PKC and the PKA pathway concurrently using a general kinase inhibitor (H7), did not provide the parasite with a significant advantage with respect to increased infection levels, over conditions where the PKC pathway was specifically impaired with PMA.

Of particular interest, we demonstrated that abrogation of calmodulin-dependent protein kinase signal transduction had an adverse effect on *Leishmania* survival within the macrophage, presumably due to a more selective toxic effect on the parasite itself. Impairment of this pathway within the macrophage did not affect macrophage cell viability, thus selective impairment of signal transduction may represent a potential strategy for the treatment of leishmaniasis.

In summary of our investigation of the effect of *L. donovani* on signal transduction in the macrophage, we established that this protozoan has evolved to selectively impair host cell biochemical processes involved in transmitting information from the cell surface to the nucleus via the PKC signal transduction pathway, and such activity had biological relevance to the establishment of infection. These studies on signal transduction in *L. donovani* served to enhance our understanding of the way in which this protozoan is able to subvert the macrophage microbicidal mechanisms.

Throughout our studies of the effect of *L. donovani* on signal transduction in the macrophage, we observed that infected cell cultures consistently contained more cells than non-infected cultures. This represented a potential novel positive influence which *Leishmania* had on the macrophage cell. While a positive effect of numerous virus

infections, including small double stranded DNA viruses, on host cell replication has previously been documented [reviewed in 17], this issue has not been addressed in a protozoan system. Thus, we undertook to characterize this positive enhancement of macrophage numbers by *Leishmania*, and to investigate the potential mechanism behind this enhancement. The results of these studies are presented in manuscripts II and III.

Our investigation revealed that infection with *L. donovani* enhanced bone marrow-derived macrophage survival in the absence of exogenous growth factor. This effect was found to be parasite specific, and required both sustained infection of the macrophage by *L. donovani*, and active protein synthesis. In addition to an enhancement of survival, infected macrophages demonstrated greater phagocytic capacity than non-infected cells in the absence of growth factor, implying that these cells were more biologically active. These observations could have relevance *in vivo*, where enhancement of infected macrophage viability could facilitate the spread of infection by increasing the number of host cells for both parasitization by *L. donovani*, and uptake by the sandfly vector. As well, enhanced phagocytic capacity of *L. donovani*-infected macrophages could result in increased parasite uptake, and heavier parasite loads.

Further investigation revealed that this positive influence by *L. donovani* on its host cell was mediated, at least in part, by a soluble factor which was secreted by infected cells into the culture medium, which enhanced macrophage viability in a manner independent of cell proliferation. This observation prompted our investigation of the effect of *L. donovani* on macrophage cell death by apoptosis, and macrophage cytokine gene expression.

In the absence of growth factor, bone marrow derived macrophages rapidly undergo rapid cell death by apoptosis [18]. Since it was shown in manuscript II, that macrophages infected with *L. donovani* demonstrated enhanced viability in the absence of growth factor, it was of interest to determine if *L. donovani* could modulate macrophage cell death. Our studies revealed that infection of macrophages with *L. donovani* promastigotes, or treatment of macrophages with LPG, inhibited cell death by apoptosis induced by the removal of M-CSF. This effect was also achieved by treatment of macrophages with *L. donovani*-infected cell supernatants, implicating the elaboration of a soluble factor by these cells as a potential mediator of this inhibition of cell death.

As macrophages represent a major source of inflammatory and growth cytokines, we investigated the cytokine gene profile of macrophages infected with *L. donovani*. This analysis revealed four cytokines whose expression was induced by infection with *L. donovani*; GM-CSF, TNF-α, TGF-ß and IL-6. Of these cytokines, only GM-CSF and TNF were demonstrated to be capable of inhibiting macrophage apoptosis *in vitro*. Quantification by ELISA revealed that while significant levels of TNF-α were elaborated into the culture cell supernatants during macrophage infection with *L. donovani*, no GM-CSF was produced. The finding that TNF-α was produced in response to infection, in addition to its ability to inhibit apoptosis *in vitro*, designated this cytokine as a candidate for the inhibition of apoptosis in *L. donovani*-infected macrophages. However, attempts to restore the ability of *L. donovani*-infected macrophages to undergo apoptosis utilizing neutralizing anti-TNF-α antibodies were unsuccessful, implying that other factors in addition to TNF-α must be involved.

It is likely that additional cellular gene products, other than those identified in our study, are expressed in infected macrophages. The products of *L. donovani*-induced genes may act intracellularly, as well as extracellularly, to collectively inhibit apoptosis. Furthermore, in additon to the induction of gene expression, an impairment of gene expression by *L. donovani* may also contribute to the inhibition of macrophage apoptosis. For example, studies from our laboratory have demonstrated that both infection with *L. donovani*, and treatment of macrophages with LPG, result in an inhibition of PKC-mediated c-*fos* gene expression [6,7]. As the PKC signal transduction pathway [19], and c-*fos* expression in particular [20], have been implicated in the process of apoptosis [18,19], impairment of these cellular functions by *L. donovani* may contribute to the impairment of apoptosis in infected macrophages. Our observation that treatment of macrophages with LPG induced a dose dependent abrogation of apoptosis in the absence of growth factor supports this argument. Thus, it was not surprising that the addition of neutralizing antibodies to any one cytokine, such as TNF-α, did not restore the ability of infected BMMs to undergo apoptosis.

In summary, we demonstrated that *L. donovani* has a positive influence on host cell survival in the absence of growth factor. The enhancement of macrophage viability in infected macrophages was due to the inhibition of host cell death, which is most likely due to the synergistic interaction of a number of factors, including both gene induction and impairment. The enhancement of host cell viability could have implications *in vivo*, as this would facilitate the spread of infection by increasing the number of host cells for parasitization by *L. donovani*, and by increasing the number of circulating infected

macrophages for uptake by the sandfly vector.

In conclusion, infection with *Leishmania* involves a balance between impairing the microbicidal activity of the host cell, yet also enhancing the viability of the infected cell. Both these effects are likely to be important for the establishment, and spread of infection.

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