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Modulation of Macrophage Functions by Components of *Entamoeba histolytica*

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Doctor of Philosophy

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ABSTRACT

Entamoeba histolytica is a protozoan parasite and the causative agent of amebiasis. Activated macrophages are the main host effector cells in host defence against *E. histolytica*, through the production of nitric oxide (NO) which is cytotoxic for the parasite. NO is upregulated by tumor necrosis factor-alpha (TNF-α) produced by macrophages. The objective of this study was to determine the effect of amebic components on TNF-α and NO production by macrophages. Soluble *E. histolytica* proteins stimulated naive macrophages for enhanced TNF-α mRNA expression through PKC signal transduction. *E. histolytica*-induced TNF-α mRNA expression was unstable, and macrophages pretreated with *E. histolytica* proteins expressed reduced levels of TNF-α mRNA in response to LPS or IFN-γ + LPS. In contrast, the purified galactose-adherence lectin (Gal-lectin) of *E. histolytica* stimulated naive macrophages for stable TNF-α mRNA expression and protein production. Furthermore, IFN-γ primed macrophages produced TNF-α and NO in response to the Gal-lectin. Naive macrophages exposed to Gal-lectin + IFN-γ were activated to kill *E. histolytica* trophozoites *in vitro* by NO. Anti-lectin monoclonal antibodies that recognize non-overlapping epitopes of the 170 kDa heavy subunit of the Gal-lectin identified amino acids 596-1082 as important in mediating amebic adherence to target cells and TNF-α mRNA induction in macrophages. Likewise, a region between amino acids 596-818 of the 170 kDa Gal-lectin, in conjunction with IFN-γ, activated macrophages for TNF-α and NO production and amebicidal activity. This research demonstrates the immunogenic potential of the *E. histolytica* Gal-lectin and the critical regions that could be used as a subunit vaccine candidate against amebiasis.

ABRÉGÉ

Entamoeba histolytica est le parasite protozoaire qui cause l'amibiase. Les macrophages activés sont les principales cellules impliquées dans la défense de l'hôte contre *E. histolytica*, en vertu de leur production d'oxyde nitrique (NO), une molécule cytotoxique pour le parasite. La production de NO est stimulée par le facteur nécrosant des tumeurs alpha (TNF-α), qui est aussi produit par les macrophages. L'objectif de cette étude était de déterminer l'effet qu'ont certains constituants de l'amibe sur la production de TNF-α et de NO par les macrophages. Les protéines solubles d'*E. histolytica* ont stimulé des macrophages naïfs à exprimer l'ARNm de TNF-α via une transmission des signaux dépendante de la protéine kinase C (PKC). L'expression d'ARNm de TNF-α induite par *E. histolytica* était instable, et des macrophages pré-traités avec les protéines d'*E. histolytica* ont exprimé des quantités réduites d'ARNm de TNF-α en réponse au lipopolysaccharide (LPS) ou à l'interféron gamma (IFN-γ) + LPS. Par contre, la lectine d'adhérence au galactose (Gal-lectine) d'*E. histolytica* purifiée a stimulé l'expression stable d'ARNm de TNF-α, ainsi que la production de la cytokine. Des macrophages naïfs exposés à la Gal-lectine + IFN-γ ont été activés à tuer des trophozoïtes d'*E. histolytica* *in vitro* au moyen de NO. Des anticorps monoclonaux qui reconnaissent des épitopes distincts de l'unité de 170 kDa de la Gal-lectine ont identifié les acides aminés 596-1082 comme étant importants pour l'adhérence des amibes aux cellules cibles, ainsi que pour l'induction d'ARNm de TNF-α chez les macrophages. De même, une région se situant entre les acides aminés 596 et 818, combinée à l'IFN-γ, a activé des macrophages à produire TNF-α et NO, et à démontrer une activité amibicide. Cette étude démontre le potentiel immunogénique de la Gal-lectine d'*E. histolytica*, ainsi que les régions importantes de la molécule qui pourraient être utilisées comme vaccin contre l'amibiase.

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I would like to thank my research supervisor, Dr. Kris Chadee, whose direction, encouragement, support and most of all, patience made this possible. Thank you for your readiness for me to present my work at conferences and publish.

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The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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STATEMENT OF ORIGINALITY

The following aspects described in this thesis are considered contributions to original knowledge:

MANUSCRIPT I:

This was the first report that demonstrated the induction of naive macrophages for enhanced transcription of the cytokine, tumor necrosis factor-alpha (TNF-α), and the proto-oncogene, c-*fos* by soluble proteins of *Entamoeba histolytica*, through protein kinase C signal transduction pathway. The TNF-α and c-fos mRNAs induced in response to the soluble *E. histolytica* proteins were rapidly degraded and the cells became refractory for stimulation with macrophage activating agents (eg. LPS or IFN-γ+ LPS). This report demonstrates that the suppressive effects of *E. histolytica* upon macrophage functions occurs at the level of RNA expression.

MANUSCRIPT II:

This was the first report demonstrating the stable mRNA expression and protein production of TNF-α by naive macrophages in response to a purified amebic molecule, the galactose-adherence lectin (Gal-lectin). This work demonstrates the immunogenic potential of the Gal-lectin. Furthermore, we identified a region between amino acids 596-1082 of the Gal-lectin which mediates *E. histolytica* adherence to target cells and the induction of TNF-α mRNA.

MANUSCRIPT III:

This was the first report that the Gal-lectin was capable of activating IFN-γ primed macrophages to produce TNF-α and NO. Also, naive macrophages exposed to Gal-lectin + IFN-γ were activated to kill *E. histolytica* trophozoites *in vitro*. Collectively, these results indicate that the amebic Gal-lectin is a potent immunostimulator and

possesses the ability to activate macrophages for amebicidal activity. A region of the Gal-lectin (aa 596-818) responsible for macrophage activation was defined and holds promise for use as a subunit vaccine candidate.

STATEMENT OF AUTHORSHIP

All of the experiments contained within the Manuscripts, were designed and performed by myself under the guidance of Dr. Kris Chadee. Dr. Kris Chadee, provided financial resources for the laboratory work, advice on the design and analysis of all the studies, and in the preparation of all manuscripts for publication.

The experimental protocols of the research composing Manuscripts II and III were contributed greatly by Dr. Barbara Mann who provided the purified amebic Gal-lectin and the anti-lectin monoclonal antibodies used in these studies. The research work was carried out by myself. Dr. Mann advised me on the preparation of the manuscripts prior to publication.

The research technician in Dr. Chadee's laboratory, Kathy Keller, was responsible for the cultivation of *Entamoeba histolytica* and provided the infrastructure support for all of the studies. She gave technical advice and suggestions for the experimental work and demonstrated the techniques for the PKC assay used.

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GENERAL DISCUSSION

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LIST OF ABBREVIATIONS

aa	amino acid
AG	aminoguanidine
AP-1	activator protein-1
BMM	bone marrow-derived macrophages
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CSF	colony-stimulating factor
DAG	diacylglycerol
E. histolytica	*Entamoeba histolytica*
ELISA	enzyme-linked immunosorbent assay
Gal-lectin	galactose-adherence lectin
IFN-γ	gamma interferon
iNOS	inducible nitric oxide synthase
$InsP_3$	inositol triphosphate
IL	interleukin
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-B
NO	nitric oxide
NO_2^-	nitrite
PGE_2	prostaglandin E_2
$PtdInsP_2$	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha

SECTION I: LITERATURE REVIEW

INTRODUCTION

Entamoeba histolytica is a protozoan parasite and the causative agent of human amebiasis. Amebiasis is the third leading parasitic cause of death worldwide (1). Chemotherapy is currently the only form of treatment available. *E. histolytica* adhere by the galactose-adherence lectin (Gal-lectin) to colonic mucins for colonization and to epithelial cells as an initial step for contact-dependent killing (2) (3,4). The Gal-lectin is a 260 kDa transmembrane glycoprotein composed of a 35 kDa light and a 170 kDa heavy subunit (5). The 170 kDa subunit mediates amebic adherence (5), is a conserved antigen and is recognized by patients' immune sera worldwide (6).

Clinical evidence and results from animal models indicate that protective immunity develops following drug-cure (7-11), which support the feasibility of developing a vaccine against amebiasis. Although patients suffering from amebiasis develop high titres of anti-amebic antibodies, there is no correlation between the levels of antibodies and clinical outcome of the disease (12, 13), suggesting that humoral immunity may be ineffective in protecting against recurrent amebiasis. In contrast, the cell-mediated immune response plays a major role in host resistance (14-19). T-lymphocytes isolated from patients or animals following drug-cure or immunization with amebic antigens or Gal-lectin produced IFN-γ and IL-2 and proliferated in response to amebic antigens or Gal-lectin (20, 21). Furthermore, macrophages exposed to the culture supernatants from these T-lymphocytes were activated to kill *E. histolytica* trophozoites *in vitro* (17, 18). Macrophages are the most important effector cells in host defence and resistance against *E. histolytica* (17, 18, 22-24). Nu/nu mice can only develop amebic liver abscesses following the depeletion of their macrophages (22). *In vitro*, macrophages activated with IFN-γ + LPS or TNF-α,

kill amebic trophozoites by nitric oxide and other non-nitrogen dependent mechanisms (23, 24). In contrast, macrophages isolated from gerbil amebic liver abscesses demonstrate suppressed effector and accessory cells functions, such as diminished respiratory burst (25), reduced secretion of TNF-α (26) and NO (27) in response to LPS and an inability to kill trophozoites (27). Collectively, these observations suggest that to be effective against *E. histolytica*, macrophages must be activated before the amebae have the opportunity to exert their immunosuppressive effect.

Before achieving the ultimate goal of developing an effective amebiasis vaccine, a greater understanding of the modulation of the host immune cells by *E. histolytica* must be undertaken. To date, vaccine research against amebiasis has failed to study the responses of macrophages which are the main host defensive cells (29, 30). The objective of this study was to investigate the effects of soluble *E. histolytica* proteins and a specific amebic molecule, the Gal-lectin, upon macrophage effector functions, specifically TNF-α and NO production and amebicidal activity. Further work was aimed at defining the region of the Gal-lectin responsible for stimulating macrophage activity. These studies were necessary as the responses induced by a potential vaccine candidate molecule must be well characterized and defined prior to being selected as an amebiasis vaccine.

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CHAPTER 1

Entamoeba histolytica

1.1 Classification and Epidemiology

The genus *Entamoeba* includes several species of human parasites: *E. histolytica* (Schaudinn 1903), *E. hartmanni* (von Provazek 1912), *E. coli* (Grassi 1879, Hickson 1909) and *E. gingivalis* (Smith and Barrett 1914) (1), but *E. histolytica* is the only important cause of disease: intestinal colitis and amebic liver abscesses (2). Schaudinn described the parasite as "histolytica" because of its ability to cause tissue destruction (3). He also distinguished two forms of *E. histolytica* that infect humans: one which was commensal and another which was associated with disease. Recently, isoenzyme analysis (4) as well as genetic (5, 6) and immunological studies (7) indicate that these two forms are two distinct species (4,5): *E. histolytica*, a pathogen which may or may not be virulent and *E. dispar*, the avirulent pathogen described by Brumpt (8), which does not invade the intestinal mucosa (4). *E. histolytica* has the following classification (9):

Kingdom	Protista
Phylum	Sarcomastigophora
Subphylum	Sarcodina
Class	Lobosea
Order	Amoebida
Family	Entamoebidae
Genus	Entamoeba
Species	histolytica

E. histolytica has a worldwide distribution, from the cold climate of Canada to the warmer climes of the Southern hemisphere, but it is endemic only in tropical countries. *E. histolytica* infects an estimated 1% of the world's population in which complications of intestinal dysentery and amebic liver abscesses may occur (10). Left untreated, amebic liver abscesses result in death of the patients. Annual estimates of 40 000-100 000 deaths attributed to amebiasis place it third, after malaria and schistosomiasis, as the most common cause of death from a parasitic disease (10). It is spread from person-to-person either by contaminated food/water or by direct fecal/oral contact. High risk groups include individuals in lower socio-economic circumstances where conditions of inadequate sanitation and a lack of purified water prevail.

1.2 Life Cycle of *E. histolytica*

Amebic infections produce a spectrum of conditions from asymptomatic cyst carriers, to those with bloody diarrhea, ulcerative lesions and progressive liver abscesses. The life cycle of *E. histolytica* begins when a person becomes infected by ingesting cysts. The circular cysts are 8-20μm in diameter and are encased in an acid-resistant chitinous cell wall (11). Besides being resistant to gastric acidity, cysts resist adverse environmental conditions and have been shown to survive in stools or water for a period of 2 months at 0°C or for 48 hrs at 48°C (12). Following ingestion, a cyst reaches the small intestine where an activation phenomenon occurs. The cyst wall is disrupted by the formation of a small opening through which a metacystic ameba emerges (11). The metacystic ameba then undergoes several divisions giving rise to a total of 8 mononucleated daughter amebae known as trophozoites (11). Trophozoites are deceptively simple-looking organisms, 10-40 μm in size, with a clear ectoplasm, a granular endoplasm with numerous vacuoles and a single nucleus (11). Trophozoites lack mitochondria, an organized rough endoplasmic reticulum and a Golgi apparatus (11). As trophozoites are non-flagellated,

motility is accomplished through the formation of pseudopods (11). Unlike cysts, trophozoites are fragile and do not survive outside the host (12). They do not remain in the small intestine but colonize the colonic lumen by multiplying and feeding on food residues and bacteria. In the colon, they either multiply by division (binary fission) or transform into cysts which are then passed out in the stool to continue the life cycle of the parasite (11).

1.3 Pathogenesis of *E. histolytica*

To date, there exists no animal model of intestinal disease which directly parallels infection in humans (ingestion of cysts or trophozoites followed by colonic disease and/or encystation with subsequent transmission of infection). However, *in vivo* models of colonic amebiasis using rodents demonstrate that prior to invasion, *E. histolytica* causes a depletion of luminal and goblet cell mucin (13-15). Trophozoites secrete a potent mucus secretagogue that is comparable in activity to purified cholera toxin (16). Erosion of the mucosal surfaces results in exposure of the epithelial cell layer which permits mucosal invasion and destruction of the epithelium.

In vitro and *in vivo* models of invasive amebiasis have indicated the importance of amebic adherence as the initial step for its cytolytic mechanisms (17). An *in vitro* model was developed to study the pathogenesis of amebiasis through the adherence of *E. histolytica* trophozoites to mammalian tissue culture cells (17). Amebic killing of Chinese hamster ovary (CHO) cells involved initial adherence, followed by cytolytic and phagocytic events (17). Adherence to target cells is an absolute prerequisite for cytolytic mechanisms and facilitates amebic invasion (15). The relevance of this model has been supported by comparative studies of the *in vitro* interaction of axenic amebae with other targets including human neutrophils, monocytes, erthyrocytes and rat and human colonic mucosa (13-15, 18).

Amebic adherence *in vitro* to human colonic mucins, rat colonic epithelial cells and mammalian tissue culture cells is mediated by the 170 kDa heavy subunit of a 260 kDa heterodimeric surface lectin (19-21). This adherence lectin was found to be inhibited by N-acetyl-D-galactosamine (GalNac), galactose (Gal), Gal-terminal oligosaccharides and rat and human colonic mucins (19-22), whereas monosaccharides such as N-acetyl-D-glucosamine (GlcNAc), mannose, sialic acid or dextrose had no effect (19, 20, 23). The Gal-inhibitable adherence lectin has been purified from *E. histolytica* and consists of a 170 kDa heavy subunit that is linked via disulphide bonds to a 35 kDa light subunit (24). The 170 kDa subunit is an antigenically conserved and immunodominant antigen whereas humoral immune responses to the 35 kDa subunit has not been detected in humans or gerbils (25, 26). The heavy subunit is predominantly responsible for mediating amebic adherence and therefore plays a central role in adhesion and contact-dependent cytolysis, as suggested by studies using monoclonal antibodies (mAbs) specific for the 170 kDa lectin subunit (27).

E. histolytica possesses multiple mechanisms by which to damage tissue, but the extent to which each mechanism contributes to the overall cytotoxicity is, as yet, undetermined. Cytotoxic mechanisms occurring during contact-dependent cytolytic events may involve an ion-channel pore-forming protein, the amebapore (28, 29). This protein damages target membranes via the membrane assembly of structural and functional pores which induce the collapse of the target cell transmembrane potential in a manner similar to the perforins produced by cytotoxic T lymphocytes and natural killer cells. Studies have implicated calcium-dependent and calcium-independent phospholipase A activities in cytolysis (30), and a cytopathic (as opposed to cytotoxic) effect of cysteine proteinases (31). While the mechanisms of contact-dependent killing by *E. histolytica* are yet undefined, it is clear that the adherence of trophozoites via Gal-lectin initiates a chain of events which leads to the rapid (within 5-15 minutes) killing of target cells.

Following disruption of mucosal epithelial cells, the secretion of cytolytic enzymes may enable ameba to destroy basement membranes in order to enter the tissue. In the submucosa, the amebae diffuse laterally causing lysis and necrosis of neighbouring cells (32). These small areas of necrosis may become confluent and form ulcers, some of which subsequently affect the muscular layer and give rise to perforations. Trophozoites in contact with blood and lymph may disseminate to any part of the body, but most commonly establish in the liver which they reach via the hepatic portal vein (32). In systemic sites such as the liver, tissue necrosis occurs with destruction of the parenchyma, resulting in the formation of amebic liver abscesses. These extraintestinal lesions are not rare as hepatic amebiasis occurs in 6-10% of patients with invasive amebiasis. Nearly half of patients with amebic liver abscess do not have a history of amebic colitis. An amebic liver abscess is sterile and only *E. histolytica* trophozoites are found within its walls. The abscess is usually a well-defined yellowish fluid-filled hepatic lesion containing neutrophils, monocytes and cellular debris and is surrounded by a very thin connective tissue capsule (32). Recent studies (23) suggest that the host inflammatory response to *E. histolytica* may contribute to the destruction of host tissues. Amebae-mediated lysis of neutrophils results in the release of large amounts of lysosomal enzymes which destroy hepatocytes and which establish the initial lesions prior to amebic liver abscess formation. The natural tendency is for an amebic liver abscess to continue growing until it ruptures. The most frequent cause of death is acute peritonitis following rupture of the amebic liver abscess into the peritoneal cavity. For reasons as yet not understood in extraintestinal amebiasis, cysts are no longer produced by amebae but rather the trophozoites continue to divide by binary fission. Therefore, *E. histolytica* represents a paradox as the infliction of harm to its host is altogether counterproductive for the normal propagation of the parasite.

In summary, the cytolethal action of amebae occurs in three steps: disruption of intestinal mucosal barriers, adherence to the intestinal mucosa/target cells, and finally contact-dependent cytolysis and phagocytosis of host intestinal and inflammatory cells, which can result in colonic ulcers and invasion of other tissues or distant organs.

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CHAPTER 2

Host Defense In Amebiasis

2.1 Immunoresistance

Unless treated, amebic hepatic abscesses grow relentlessly until they are fatal to the host. It appears, therefore, that during an initial infection the host immune response is ineffective in controlling the spread of the parasite. However, clinical experience suggests that patients drug-cured of amebic liver abscess rarely have recurrent invasive amebiasis (1,2). A single, uncontrolled study in Mexico City demonstrated that only 0.29% of 1, 021 patients, who were treated for amebic liver abscesses and who were at a high risk to reacquire the disease, suffered a recurrence of hepatic abscesses within five years (1); this is less than the expected rate for the total population (3,4). Furthermore, protective immunity developed in animal models following cure of invasive infection or after immunization with amebic proteins (5-10). Therefore, it would appear that acquired immunity develops after invasive infection has been cured, supporting the feasibility of vaccine development. No vaccine, however, is currently available for the prevention of invasive amebiasis.

2.2 Mucosal Defense

The secretion of mucins in the colon is the first line of defense against invasion by *E. histolytica* trophozoites (11-13). Mucins are macromolecules ($M_r > 1 \times 10^6$) consisting of a protein core covered by a dense layer of oligosaccharide side chains (>80% w/w) (14, 15). The viscous and gel-forming properties of mucins prevent intestinal pathogens from adhering to and destroying the underlying epithelial cells (16, 17).

Adherence of trophozoites to epithelial cells is an absolute prerequisite for the initiation of amebic invasion in the colon (12, 18) and parasite cytolytic activity (19). Adherence of *E. histolytica* to colonic epithelial cells is mediated through the 170 kDa heavy subunit of the amebic Gal-lectin molecule, and can be inhibited by the addition of galactose or galactose-containing oligosaccharides (12, 20). Purified rat and human colonic mucins bind with high affinity (dissociation constant 8.20×10^{-11} M^{-1}) to the Gal-lectin (11, 12). Colonic mucins provide a defense mechanism against invasion by binding to the Gal-lectin and thereby inhibit both the amebic adherence to colonic cells and the subsequent cytolysis of these cells by the parasite (11, 12). However, high levels of mucin secretion may eventually facilitate the pathogenesis of invasive amebiasis. In experimental animal models the invasion of the colonic mucosa by *E. histolytica* was preceded by the depletion of luminal and goblet cell mucins (19, 21) resulting in the exposure of the epithelial cell layer. Therefore, the potent mucus secretagogue effects exhibited by *E. histolytica* trophozoites may function to diminish the protective mucus layer of the host (21).

2.3 Humoral Immunity

The contribution of humoral immune mechanisms to resistance to invasive amebiasis is unknown. During invasive amebiasis, anti-amebic secretory IgA is produced at mucosal surfaces (22), and recently it was demonstrated that purified rat secretory IgA against the Gal-lectin inhibited amebic adherence to mammalian cells *in vitro* (23). However, it is not known what role IgA plays in controlling or eliminating *E. histolytica* from the colon.

Following invasion, it has been observed that 95% of patients with liver abscesses develop circulating IgG antibodies specific for *E. histolytica*, with titres of 1:128 or higher (2, 24). The majority of antibodies (> 90%) recognized the Gal-lectin of *E. histolytica*

(25). This antibody response is long lasting and has been shown to persist for more than 3 yrs (26, 27). Nevertheless, in humans there is no correlation between high anti-amebic antibody titres and the clinical outcome of the disease (28). In contrast, in the SCID mouse model, passive transfer of rabbit anti-amebic sera resulted in complete protection against amebic liver abscess in 58% of the mice, but the mechanism of this protection was not defined (29). Although antibodies bind to amebae (30), they are not effective in opsonization since amebae can ingest or shed attached antibodies without loss of parasite viability (31-34). This latter phenomenon, known as "capping", occurs when bound antibodies are clustered by the parasites into small patches on their surfaces, then the patches are rapidly mobilized to the posterior pole of the cell and released into the extracellular medium as membrane vesicles without any damage to the amebae (31, 32, 34). "Capping" in *E. histolytica* has been suggested to be a mechanism for evasion of the host humoral immune response by the parasite as rapid mobilization and release of surface antigen/antibody complexes would interfere with antibody-dependent complement lysis of the parasite.

In summary, the humoral immune response following invasion of *E. histolytica* apparently neither cures the infection nor prevents reinfection.

2.4 Cell-Mediated Immunity

Present evidence in both human and animal models indicates that acquired resistance to invasive amebiasis is associated with the development of an effective cell-mediated immune response (35-41).

Neutrophils may be the first immune cells to interact with *E. histolytica* trophozoites *in vivo*. In animal models of amebiasis, a rapid neutrophilic response to the presence of *E. histolytica* trophozoites in tissues (42, 43) occurred as a result of the

chemotactic properties of amebic membrane bound proteins (44). Neutrophils in contact with *E. histolytica* trophozoites were rapidly killed or were phagocytosed by the parasites (45, 46). The lysosomal enzymes released from the dead neutrophils destroy hepatocytes adjacent to amebae and may contribute to the early formation of amebic liver abscesses (42, 43). However, human neutrophils activated by IFN-γ and TNF-α can kill *E. histolytica* trophozoites *in vitro* (44). These observations suggest that neutrophils, when lysed, may contribute to the pathology of amebiasis, but at the same time, when activated by cytokines, neutrophils may have the potential to prevent the dissemination of the parasite to tissues.

Lymphocytes isolated from uninfected patients or those with early hepatic lesions are lysed by *E. histolytica* trophozoites *in vitro*. In contrast, lymphocytes from patients recovering from amebic hepatic abscess can readily destroy amebae (38). In clinical studies, T-lymphocytes isolated from patients cured of amebic liver abscess demonstrated an antigen specific proliferative response to and cytotoxic activity against *E. histolytica* trophozoites (38). Furthermore, in response to either amebic proteins (38-40) or Gal-lectin (47), these lymphocytes produced lymphokines, including gamma-interferon (IFN-γ), which activated human monocyte-derived macrophages to kill *E. histolytica* trophozoites *in vitro* (38-40). Similarly, *ex vivo* studies demonstrated that splenocytes from lectin-immunized gerbils produced IL-2 and IFN-γ in response to Gal-lectin (48), suggesting the involvement of a T_H1 response in protective immunity. Moreover, when lectin-immunized gerbils were challenged with *E. histolytica* trophozoites, the splenocytes harvested from these animals demonstrated proliferative and amebicidal responses as well as produced IFN-γ in response to the Gal-lectin, indicating that the immunization with Gal-lectin was effective *in vivo* (48). The main contribution of T-lymphocytes in host defense against *E. histolytica* is through the production of lymphokines, notably IFN-γ, which activate macrophages for amebicidal activity.

Nude mice sustained an amebic infection only after being depleted of their macrophages by treatment with silica (41). This observation highlights the importance of macrophage function for host immunity. Spleen and peritoneal macrophages isolated from infected or immunized animals demonstrate increased amebicidal activity (49). *In vitro* studies have shown that murine macrophages activated with either IFN-γ and LPS, or TNF-α kill *E. histolytica* trophozoites in a contact-dependent manner (35). Amebic killing was not the direct result of TNF-α or IFN-γ cytotoxicity towards the parasite (37) but rather through the synergistic action of both molecules in activating macrophages to induce nitric oxide (NO) production (50), cytolytic proteases and H_2O_2 (35). NO is the major effector molecule produced by activated macrophages to kill *E. histolytica* trophozoites *in vitro*, as demonstrated by the abrogation of macrophage amebicidal activity in the presence of the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA), or by the depletion of arginine from the medium by the addition of arginase (50). Recently, it was demonstrated that antiserum to TNF-α suppressed not only TNF-α secretion, but also NO production and amebicidal activity in activated macrophages (51). TNF-α is central for NO production by elevating the levels of the inducible nitric oxide synthase (iNOS) mRNA in macrophages (51). As observed with lymphocytes (38) and neutrophils (45, 46), macrophages can also be lysed by amebic trophozoites (40). This suggests that although macrophages are potent effector cells against *E. histolytica*, they must be activated with a combination of stimuli/cytokines to increase their amebicidal capacities.

In summary, it appears that the cell-mediated arm of the host immune defense, specifically the induction of T_H1 cytokines and the subsequent activation of macrophages, is necessary for limiting the spread of the parasite and preventing recurrent invasive amebiasis.

2.5 Immunosuppression in Amebiasis

The inability of the host to control an initial infection of *E. histolytica* without the aid of chemotherapy suggests a suppression of the host immune response. During acute disease in humans there is a depression of cell-mediated immunity as demonstrated by a decreased number of helper (CD4+) T-lymphocytes in peripheral blood and decreased T-lymphocyte proliferative responses to amebic antigens (38, 52). Similarly, amebic proteins inhibited the blastogenic response of murine splenic lymphocytes to LPS and concanavalin A (con A) *in vivo* and in *vitro* (53). Taken together these observations suggest that amebic components can affect lymphocyte functions.

Macrophages are also susceptible to the suppressive effects of *E. histolytica* components. Macrophages isolated from amebic liver granulomas in gerbils demonstrated a decreased production of TNF-α (54), NO (55), H_2O_2 (56), IL-1 (56) and Ia antigen expression (57). They were also unresponsive to lymphokine stimulation to kill amebae or P815 tumor cells (55). Furthermore, while TNF-α is produced constitutively at low levels by amebic liver abscess-derived macrophages, it was not detected in the serum of patients or animals with invasive amebiasis, indicating the presence of a local inflammatory response at the site of the granuloma (54). Amebic liver granuloma macrophages at the margin of the amebic lesions are continually exposed to excretory and secretory amebic components as well as whole amebic cell lysates, thus there is ample opportunity for amebic molecules to modulate macrophage functions. In contrast, macrophages at sites distal to the abscess (peritoneal, spleen) are not suppressed and have competent macrophage functions (49, 54, 56). These studies suggest that immunomodulation is a local event associated with macrophages in close proximity to the parasites.

A similar down-regulation of macrophage functions was observed *in vitro*, when elicited peritoneal macrophages exposed to soluble amebic proteins produced decreased levels of H_2O_2 and IL-1 in response to LPS (56). Furthermore, naive peritoneal macrophages exposed to *E. histolytica* proteins prior to LPS stimulation showed a marked decrease in TNF-α (54) and NO production (55), Ia antigen expression (57), and respiratory burst potential (58). In contrast, treatment of macrophages with amebic proteins increased the production of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) (59). PGE_2 has potent immunosuppressive effects on immune cells including inhibition of macrophage/monocyte functions, T-lymphocyte activation and lymphokine production (60, 61). The stimulation of eicosanoids (PGE_2, LTC_4) by amebic components may contribute to the mechanism by which *E. histolytica* suppresses cell-mediated immune responses during the course of the infection.

In summary, while activated macrophages demonstrate potency for amebic killing (35, 37-41) and appear to control the infection in an immunized host, *E. histolytica* components can downregulate macrophage effector and accessory cell functions (54-57) at the site of the lesions.

2.6 References

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CHAPTER 3

Activation And Functions Of Macrophages

3.1 Biology of Macrophages

Macrophages are mononuclear phagocytes which belong to a family of tissue and blood derived cells that exhibit diverse functions. Mononuclear phagocytes arise from precursors in the bone marrow and, after circulating briefly in the blood as monocytes, they immigrate to tissues where they develop into macrophages (1,2). Macrophages are widely distributed in tissues including the spleen, lungs, liver, brain, bone, peritoneum and renal glomeruli (3). Unlike neutrophils, mononuclear phagocytes are not "end cells" when they exit the bone marrow (4), but rather they are still capable of division and can mature further (4) by a process commonly described as activation (5).

Mononuclear phagocytes are extremely versatile. They are powerful secretory cells with the potential to release over 100 distinct molecules (4), such as colony-stimulating factors (6), proteases (7), reactive oxygen intermediates (ROI) (8-10), reactive nitrogen intermediates (RNI) (11, 12), metabolites of arachidonic acid (13, 14), enzyme inhibitors (4), coagulation factors (15), components of the complement cascade (4) as well as other proteins, lipids and small molecules (4). Mononuclear phagocytes are extremely pinocytotic and possess cell surface receptors which enable them to recognize foreign material (4). Macrophages have an ingestion rate such that the equivalent of their entire surface area is endocytosed in 30 minutes (16) which illustrates their well merited designation of "professional phagocytes" (4). In short, macrophages collect, detoxify and contain or rid the body of unwanted molecules and cells.

Besides their role as scavenger cells, macrophages are involved in regulating the immune response (17, 18). As antigen-presenting cells, macrophages ingest immunogens and degrade them to immunogenic peptides, further bind the peptides to Class II MHC molecules and then display the peptide/Class II MHC complexes on their surface such that the peptide is now in a form recognizable to T lymphocytes (19-21). Furthermore, macrophages can regulate their own functions as well as those of other immune cells through the secretion of cytokines such as IL-1 (22), TNF-α (23, 24), and IL-6 (4) and inflammatory mediators such as prostaglandins (13) and leukotrienes (14).

Macrophages can kill invaders by antibody-dependent cellular cytotoxicity mechanisms (25, 26). Opsonized particles are bound by macrophages through their surface receptors which recognize either the Fc portion of IgG antibodies (27, 28) or components of complement (29). Antibody-independent mechanisms of macrophage killing of tumor cells or invaders are mediated through the release of molecules, such as TNF-α (23, 30-34) and NO (11, 12, 35).

While macrophage activation has been traditionally designated as the development of microbicidal or tumoricidal functions (5), it has also been defined as the acquisition of competence to perform any complex function (4, 32). Macrophage activation proceeds through a cascade of events, beginning with the recruitment of young mononuclear phagocytes to a site of inflammation where they receive a priming signal (1, 2, 4). These young inflammatory mononuclear phagocytes have been termed responsive macrophages as, in comparison, resident tissue macrophages are poorly responsive to such signals (4). Responsive macrophages are characterized at the cell-surface level by the increased numbers of certain types of Fc receptors and transferrin receptors (essential for proliferation), the mobility of the C3 receptor and the expression of the mannose receptor (4). With regard to function, responsive macrophages are capable of chemotaxis,

phagocytosis, and proliferation (4).

At the site of inflammation responsive macrophages interact with a class of lymphokines called macrophage activating factors (36) which induce the next stage in activation, primed macrophages. Extensive evidence indicates that IFN-γ is a principal priming factor for macrophage activation both *in vivo* and *in vitro* (37, 38), but IFN-α/β, IL-1, IL-4, TNF-α and GM-CSF can also act as priming factors (4). Primed macrophages are characterized by an increased expression of Class II MHC (Ia) molecules, lymphocyte function-associated antigen-1 (LFA-1), and decreased expression of transferrin receptors on their surfaces (4). Primed macrophages are capable of binding tumor cells in the absence of antibodies and of presenting antigen to T lymphocytes; however, their ability to proliferate is diminished (4). These macrophages are also capable of ROI release (8, 9, 39, 40), but do not actively secrete molecules such as IL-1 or TNF-α (4). Instead, they have now acquired a sensitivity to a second signal, such that in response to this second signal, the macrophages are activated for a heightened secretory response of molecules such as TNF-α and IL-1. This second signal can be the lipid A component of bacterial lipopolysaccharide (LPS) (41), lymphokines, supernatants of tumor cells, heat-killed Gram positive microorganisms (5) or liposome-encapsulated muramyl dipeptides (4). Activated macrophages are characterized by their tumoricidal/microbicidal activity, and their ability to secrete cytolytic proteases, TNF-α, NO and ROI (32, 33, 34, 35). They have lost their ability to proliferate and their capacity for presentation of antigen to lymphocytes is reduced to a level below that expressed by primed macrophages (4). Therefore, the process of activation involves changes in macrophage functions and characteristics.

As the protective role of macrophages must be balanced delicately with their potential to cause injury and destruction of host tissues, macrophages must be appropriately regulated (32). Several components such as LPS, TNF-α and IFN-α/β have

either inductive or suppressive effects depending on the particular form of macrophage activation examined (4). A partial list of suppressive agents includes: α2 macroglobulin protease complexes, PGE_2, transforming growth factor-beta (TGF-β), high oxygen or glucose concentration and immune complexes (4, 32). The antiprotease α2 macroglobulin suppresses secretion of neutral and cytolytic proteases, the respiratory burst of macrophages, tumor cytotoxicity and Ia antigen expression (32). PGE_2 increases intracellular cAMP levels which in turn, suppress both anti-tumor functions and antigen presentation by the macrophages (42). The destructive powers of macrophages are so potent that if they are not regulated, these cells can contribute directly to chronic destructive and/or autoimmune diseases such as multiple sclerosis or rheumatoid arthritis.

3.2 Intracellular Regulation of Activation

i) Signal Transduction Pathways

The process of macrophage activation begins with the binding of a stimulator, such as LPS (43), to the macrophage cell surface receptor(s) and the subsequent transmission of this extracellular signal across the plasma membrane and through the cytoplasm to the nucleus for the activation of genes. This transmission is accomplished by second messenger pathways and signal transduction. In mouse peritoneal macrophages, stimulation with LPS results in the hydrolysis of a quantitatively minor phospholipid species, phosphatidylinositol 4,5 biphosphate ($PtdInsP_2$), by phospholipase C which leads to the generation of inositol 1,4,5 triphosphate ($InsP_3$) and diacylglycerol (DAG) (44, 45). $InsP_3$ and DAG are second messengers that activate two independent but parallel signal transduction pathways by altering the intracellular environment (46).

Following its generation from $PtdInsP_2$ in the plasma membrane, $InsP_3$ is released into the cytosol where it liberates calcium (Ca^{2+}) from intracellular stores, and causes a transient increase in the cytoplasmic free Ca^{2+} (47, 48). This increase, in turn, triggers

calcium-dependent events, such as the activation of calmodulin (CaM) kinase which phosphorylates a subset of cellular proteins (49).

The other second messenger, DAG, can be made under many different circumstances and has the potential to behave differently, depending upon its cellular location. In unstimulated cells, DAG is a normal metabolic intermediate in lipid synthesis and does not serve as a second messenger (50). However, in LPS-stimulated cells, DAG, which is formed from the hydrophobic backbone of the cleaved $PtdInsP_2$, remains within the cellular membranes where it performs as a second messenger. One means by which DAG participates in signal transduction is through the activation of protein kinase C (PKC) (51, 52), an enzyme that plays an important role in the activation of macrophages by LPS (52). DAG can also stimulate another enzyme, sphingomyelinase (SMase) which mediates the hydrolysis of sphinogmyelin to ceramide (53), another second messenger that activates a different PKC isoform (zeta) than those activated by DAG (54). The sphingomyelin/ceramide pathway has been implicated in cell signalling in response to the cytokines TNF-α (55, 56) and IL-1 (57).

In LPS-stimulated cells, DAG increases the affinity of PKC for Ca^{2+}, thereby rendering PKC active over a micromolar Ca^{2+} range (58, 59). PKC translocates from the cytosol to the membrane (60) where it forms a complex with Ca^{2+}, phosphatidylserine and DAG (61). The formation of this complex results in the tight binding of PKC to the membrane and the activation of PKC.

Similar to CaM kinase, activation of PKC results in the phosphorylation and subsequent regulation of a set of specific proteins (61) despite its close association with the membrane (49). For example, PKC phosphorylates the cytoplasmic protein Inhibitor-κB (IκB), a molecule that binds to the ubiquitous transcription activating factor, nuclear factor-κB (NF-κB) (62, 63) and by doing so, renders NF-κB inactive. . Upon

phosphorylation of IκB, NF-κB is released and immediately translocates to the nucleus where it binds to its DNA recognition sites to mediate the transcription of genes such as the ones encoding TNF-α or IL-6 (64). This mechanism of phosphorylation by activated PKC permits an extracellular stimuli, such as LPS, to modulate nuclear events through the control of the subcellular localization of a transcription activating factor.

PKC activation also increases the mRNA levels of two "primary response genes" (65), c-*jun* and c-*fos* (66, 67, 68). Both of these genes are proto-oncogenes: c-*jun* is the normal cellular homologue of v-*jun*, the oncogene of avian sarcoma virus ASV17 (69) and c-*fos* is the cellular homologue of the transforming gene of FBJ-osteosarcoma virus (70, 71). Their protein products, FOS and JUN, form a dimeric complex known as Activator Protein-1 (AP-1) (72, 73), which binds to DNA recognition sites within the promoter regions to mediate elevated transcription of many genes (74, 75, 76). Furthermore, PKC can also phosphorylate the serine/threonine residues of other kinases, such as mitogen-activated protein kinases (MAP kinase pp54 and pp42/44) which in turn are responsible for the positive regulation of the transacting activity of JUN (77, 78).

The purpose of the divided signal pathways mediated by DAG and $InsP_3$ is to generate a diverse repertoire of intracellular events which provide the cell with the versatility necessary to control a wide range of cellular processes in response to cell surface signals. These two second messengers may contribute to the final response by acting either co-operatively or synergistically and the relative importance of each pathway may vary with time. It has been proposed that high levels of intracellular calcium were responsible for the initiation of a response whereas DAG was important in the maintenance of the response (49). Conversely, one arm of the pathway may be activated without the activation of the other so as to regulate separate processes. For example, the hydrolysis of phosphatidylinositol-4-phosphate instead of $PtdInsP_2$ would yield only DAG

and the subsequent activation of PKC, without the formation of $InsP_3$ and the subsequent effects of its pathway (49). Another option is synergy between the two messenger systems. In blood platelets, individual treatment with either a synthetic diacylglycerol (1-oleoyl-2-acetyl-glycerol, OAG) or calcium ionophore has little effect on the cells, but when these two stimuli are added simultaneously, a strong secretory response of serotonin (79) and lysosomal enzymes (80) occurs. In short, these two signal pathways appear to be able to function either in an individual or synergistic manner.

In contrast to the LPS-stimulated PKC signal transduction mechanism, the second messenger pathways for growth factors (CSF-1) and cytokines (IFN-γ, IL-6) are mediated through tyrosine phosphorylation of transcription factors (reviewed in 81). For example, the stimulation of the IFN-γ receptor elicits the activation of two members of the Janus kinase family, JAK1 and JAK2. JAK1/2 are receptor-associated kinases whose activation is associated with their own phosphorylation on tyrosine residues (82). IFN-γ activity requires the activation of both JAK1 and JAK2 (83, 84). IFN-γ-activated JAK1/2 phosphorylate tyrosine residues on a cytoplasmic proteins, p91. The activated p91 (STAT1) then translocates to the nucleus where as a dimer binds directly to IFN-γ activation site elements in the promoter regions of some genes (eg. high affinity IgG receptor) and induces transcription (85,86).

Whichever signal transduction pathway is utilized, the end result is the induction of responsive genes in the nucleus through the binding of transcription factors, such as NF-κB, AP-1 or STAT1.

ii) c-*fos*

One of the earliest genetic changes elicited by many mitogens is the induction of the primary response gene expression (66, 87-89), so named because their induction proceeds in the absence of *de novo* protein synthesis (65). One prominent member of the

primary response gene family is the proto-oncogene c-*fos*, whose protein product can function with another proto-oncogene product, JUN, to form the transcription activator, AP-1 (72-74). Expression of c-*fos* is associated with the differentiation and proliferation of mononuclear phagocytes (90) as well as functional activation of macrophages by LPS stimulation (91-93). In macrophages, transcription of the c-*fos* gene can be induced by stimulation of both PKC-dependent and PKC-independent second messenger pathways which control the functional activity of the cells (94). Transcription of the c-*fos* gene, when mediated by PKC-stimulating agents (67), is induced very rapidly and is transient (68, 89), returning to its initial level within 30 minutes following induction. Nuclear run-on assays demonstrated that increased transcription of the c-*fos* gene accounted for increased c-*fos* mRNA levels observed in response to external signals (92). Meanwhile other agents such as cAMP stimulate a different protein kinase, protein kinase A (PKA), which also induces the stable transcription of c-*fos* for up to 6 hours (67, 95). The differential kinetics of c-*fos* gene expression renders this proto-oncogene a useful marker to indicate whether PKC or PKA activation occurs in response to various stimuli.

One level of the regulation of the c-*fos* gene is post-transcriptional and involves mRNA stability (96). c-*fos* gene contains in its 3' noncoding sequence a 67 bp AT-rich region which, upon removal, results in the transformation of c-*fos* into its oncogene form (97). This AT-rich region is transcribed into multiple AUUUA motifs which lead to rapid translation-linked degradation of the c-*fos* mRNA (98), and indeed, removal of this region substantially prolongs the half-life of c-*fos* mRNA (99). c-*fos* mRNA degradation requires ongoing protein synthesis and occurs in two steps: the removal of the poly(A) tail, which functions to enhance mRNA stability (100), followed by the rapid degradation of the remaining, now labile, message (101). Both actions in c-*fos* mRNA degradation are facilitated by the UA-rich sequences (101). In addition, FOS protein is capable of

inhibiting the transcription of its own gene (102).

In summary, the *c-fos* gene is carefully controlled, most likely as its gene product is involved in the regulation of other genes.

3.3 Functions of Activated Macrophages

i) TNF-α

Macrophages produce and secrete cytokines which have potent pleiotropic effects within the host. One such multifactorial cytokine is tumor necrosis factor-alpha (TNF-α). TNF was first identified and christened in 1975 when it was observed that bacillus Calmette-Guérin (BCG)-infected mice treated with endotoxin produced a serum factor that caused "hemorrhagic necrosis" of some neoplastic cell lines (23). Later, it was classified as TNF-α so as to distinguish it from lymphotoxin (TNF-β). Today the name tumor necrosis factor should be reassessed as it has become clear that it emphasizes only a single facet of the activities of this molecule. Besides its ability to selectively lyse certain transformed cells (103), TNF-α can also stimulate the proliferation of normal and neoplastic cells and act as a differentiation factor for certain leukemias. However, the major role of TNF-α is to mediate protection against parasitic, bacterial and viral infections (104). TNF-α contributes to the combat of infection through multiple mechanisms: TNF-α stimulates transcription of the proto-oncogenes c-*fos* and c-*jun*, is chemotactic for macrophages and neutrophils, activates neutrophils and platelets, enhances the killing activity of macrophages and NK cells, stimulates fibroblast and endothelial cells, regulates T and B cell immune responses, stimulates several cytokines (IL-1, IL-6, IL-8, M-CSF, and GM-CSF) from different cells, induces Class I and Class II MHC molecules, induces the cell adhesion molecules ELAM-1 and ICAM-1 and stimulates collagenase and prostaglandins (reviewed in 105).

The amino acid sequence of TNF-α is conserved between species with 79% shared identity between human and mouse (24, 106), and greater than 70% between human and rabbit (107). This conservation between sequences is consistent with the observed lack of species specificity in response to TNF-α administration. The TNF-α gene is a single copy gene and is closely linked to the cluster of major histocompatibility complex (MHC) genes, located on human chromosome 6 (108, 109) and on murine chromosome 17 (110). Mature human TNF-α consists of 157 amino acids and is not glycosylated while murine TNF-α consists of 156 amino acids and has one N-glycosylation site. Under denaturing conditions, TNF-α has a molecular weight of 17 kDa. The 17 kDa TNF-α subunit is derived by proteolytic cleavage of a 26 kDa integral membrane form of TNF-α (105). Each 17 kDa monomer has a wedgelike shape, and the native 52kDa TNF-α is formed when three 17 kDa subunits associate noncovalently into a compact tetrahedron (111).

Lymphocytes, mast cells, polymorphonuclear leukocytes, keratinocytes, astroyctes and microglial cells, smooth muscle cells, intestinal Paneth's cells and tumor cells all release TNF-α in response to various stimuli (reviewed in 112); however, activated macrophages stimulated by LPS are by far the largest source of TNF-α (112). TNF-α is not produced by macrophages constitutively. Instead, macrophages need to be activated and then TNF-α is synthesized *de novo* as it does not exist in a stored form (113). The induction of the TNF-α gene expression involves a number of signal transduction pathways, including phospholipase C (114), phospholipase A_2 (115) and protein kinase C (116, 117). W7, an inhibitor of calmodulin-dependent kinase, does not affect TNF-α mRNA levels, demonstrating that calmodulin kinase does not have a role in upregulating TNF-α in response to LPS, while, in contrast, the presence of a PKC inhibitor, H7 (1-(5-isoquinolinesulfonyl)-2-methylpeperazine hydrochloride) decreased TNF-α mRNA levels in response to LPS. (116).

Following LPS stimulation, TNF-α protein release is regulated primarily by a prolonged period of transcription (118-120) and increased translation (121). The TNF-α promoter region contains many regulatory elements involved in the control of its transcription in response to various stimuli. For example, the murine TNF-α promoter region contains sequences similar to the κB enhancer of the immunoglobulin genes which binds NF-κB (120, 122, 123). In addition, located within the TNF-α promoter is a sequence very similar to the c-*jun*/AP-1 binding site consensus sequence associated with the promoters of phorbol ester-inducible genes (124).

In response to LPS, TNF-α mRNA levels rise rapidly by a factor of 50 to 100 within the cell (118) and are maximally expressed within 90 minutes (125). The expression of TNF-α mRNA is transient. The presence of cycloheximide, an inhibitor of protein synthesis, further enhances the accumulation of TNF-α mRNA in response to LPS, suggesting the involvement of repressor proteins in the control of LPS-stimulated TNF-α transcription (126, 127). Post-transcriptional control of TNF-α depends upon the TTATTTAT sequence within the 3' untranslated region of the TNF-α gene, similar to the c-*fos* proto-oncogene. The presence of this sequence in the mRNA is associated with accelerated degradation of and interference with the translation of the mRNA (128, 129). Furthermore, murine macrophages contain a ribonuclease that selectively degrades mRNA possessing this sequence (130). This reduction of TNF-α mRNA half-life is a necessary control mechanism so as to prevent an inappropriate persistent production of a large amount of biologically active polypeptide.

In addition to LPS, other stimuli induce TNF-α production from monocytes/macrophages including phorbol esters (131) and other cytokines such as IL-2 (132), and GM-CSF (133). Furthermore, bacteria and parasites, or components thereof, such as *Plasmodium falciparum* antigens (134), *Candida albicans* (135), components of

Mycobacterium tuberculosis (136) and species of mycoplasma (137, 138) have been shown to induce TNF-α production by macrophages. Additionally, IFN-γ treatment renders macrophages exquisitely sensitive to very low amounts of LPS and amplifies both LPS-induced TNF mRNA accumulation (139) and TNF-α protein release (140). IFN-γ is potent enough to revert the defective response of macrophages from C3H/HeJ mice to LPS, and is capable of overcoming the inhibitory effect of cAMP on TNF-α production (141).

Following stimulation, monocytes secrete TNF-α in quantities greatly exceeding cell associated levels, indicating that the majority of TNF-α is secreted. Virtually all cells can respond to TNF-α because receptors for TNF have been detected on almost every cell type with the exceptions of erythrocytes and unstimulated T cells. Two TNF receptors have been cloned, in both human (142, 143) and mouse (144) which are capable of binding both TNF-α and TNF-β with comparable affinities. The extracellular domains of these two receptors are similar but these receptors differ both in size, 55 kDa and 75 kDa, and in their transmembrane and intracytoplasmic domains (142-144).

Aside from being the main producers of TNF-α, monocytes and macrophages are also one of its main targets. TNF-α is chemotactic for monocytes (145) and also acts as an autocrine inducer of its own expression (146, 147) by monocytes/macrophages. These observations, together with the fact that TNF-α can favour local leukocyte emigration through the induction of adhesion molecules on endothelial cells, suggest that the release of TNF-α by macrophages may induce further accumulation of macrophages. TNF-α can also induce the differentiation of immature precursors into monocytes, and prevent monocyte death by apoptosis, perhaps by the induction of colony-stimulating factor (CSF-1)(148).

An augmented capacity of macrophages to produce TNF-α reflects a heightened macrophage activation/maturation state (149), and TNF-α, itself, can activate macrophages for cytotoxic activities (150). Activated macrophages possess two main cytotoxic pathways: the superoxide pathway which produces ROI (H_2O_2, O_2^-) and the L-arginine dependent nitric oxide (NO) pathway which produces RNI (NO, NO_2^-). These two pathways contribute to the parasiticidal and bactericidal effects of macrophages on *Listeria monocytogenes* (151), *Leishmania major/donovani* (152, 153), *Trypanosoma cruzi* (154), *Mycobacterium avium* (155) and *Schistosoma mansoni* larvae (156), and *Entamoeba histolytica* (157).

TNF-α has a beneficial role when produced locally and transiently. However, elevated and sustained production of TNF-α can produce deleterious effects on the host, including shock in bacterial sepsis, wasting of muscles and excessive weight loss (158). Excessive TNF-α production has been implicated in chronic inflammatory reactions of certain autoimmune diseases such as multiple sclerosis (159), rheumatoid arthritis (160). TNF-α may be fatal in parasitic diseases such as murine cerebral malaria (161).

Once the immune system is triggered, there is a rapid burst of TNF-α synthesis followed by its decreased production. The host apparently makes a considerable effort to protect itself from the toxic effects of excessive TNF-α production, by tight regulation of the level of TNF-α. Inducers of cAMP formation such as forskolin and dibutyrl cAMP itself, suppress both TNF-α production and TNF-α transcript accumulation in LPS-stimulated macrophages (162). PGE_2, which is produced by macrophages in response to TNF-α, acts at the transcriptional level to inhibit LPS-induced TNF-α gene expression in macrophages (163) by increasing cAMP (164). Dexamethasone has been reported to completely inhibit LPS-induced production of TNF-α by murine macrophages (118) and to greatly reduce TNF-α production by human monocytes (165). IL-10 blocks TNF-α

production by IFN-γ/LPS-treated macrophages at the levels of both RNA and protein synthesis (166). In contrast, the immunosuppressive drug, cyclosporin A, inhibits production of TNF-α without depressing TNF-α mRNA levels (167). Infectious agents can also inhibit TNF-α through different mechanisms. For example, macrophages infected with *Toxoplasma gondii* release transforming growth factor-β (TGF-β) which downregulates TNF receptors on the surface of the infected macrophages (168), while human monocytes infected with measles virus demonstrate a reduction in the stability of LPS-induced TNF-α mRNA which leads to a reduction of TNF-α protein as compared to uninfected cells (169). In general, inhibitors of TNF-α can function by blocking at either the level of TNF-α gene transcription, translation, secretion or expression of TNF-α receptors.

In summary, during an infection, TNF-α is predominately produced by macrophages in response to exogenous stimuli and acts to increase the cytotoxic activities of macrophages. The level of TNF-α production is tightly regulated by other endogenous mediators generated during an immune response, so that the host can achieve the beneficial effects of TNF-α and yet prevent the overproduction of TNF-α which may lead to an exacerbated disease state.

ii) Nitric Oxide (NO)

Activated macrophages inflict injury on a variety of target cells through various mechanisms. One such mechanism is the formation of RNI, specifically the release of a free radical gas, nitric oxide (NO). NO cripples tumor cells through the removal of a labile iron atom from the iron-sulphur prosthetic group of the enzymes involved in mitochondrial respiration, the citric acid cycle and DNA synthesis (170). NO mediates its antibacterial effects under conditions when both ROI and RNI are induced where it reacts with superoxide to generate peroxynitrite anion which decomposes to a strong oxidant,

the hydroxyl radical (171). Under these same conditions, superoxide is rapidly dismutated to hydrogen peroxide which also combines with NO to generate a highly reactive singlet oxygen (172). These reactive oxygen species mediate the fragmentation of DNA and the oxidization of lipids and proteins which are then subsequently degraded. It is through the production of NO, or related nitrogen oxides derived from L-arginine, that cytokine-activated murine peritoneal and bone marrow-derived macrophages mediate the destruction of many parasites, such as *Leishmania major* (173, 174), *Leishmania donovani* (175), *Plasmodium falciparum* (176), *Schistosoma mansoni* (177), *Trypanosoma cruzi* (178), *Toxoplasma gondii* (179), and *E. histolytica* (157). In living organisms, the oxidation of a guanidino nitrogen of L-arginine (180), which yields nitric oxide and L-citrulline (181) is catalyzed by the enzyme, nitric oxide synthase (NOS). NOS enzymes are homodimeric hemeproteins containing FAD and FMN, and require tetrahydrobiopterin (BH_4) and NADPH for activity (182, 183). Three mammalian NOS genes have been identified (184-186), two of which code for enzymes that are constitutively expressed, and in the presence of elevated Ca^{2+}, are bound to and activated by calmodulin (187, 188). These enzymes are labelled ecNOS and ncNOS as they are expressed in the endothelium and in neurons, respectively. These are stereotypical classifications as ecNOS occurs in a variety of neuronal structures in the brain not only in blood vessels, and there have been situations, such as following damage to peripheral or central neurons, whereby ncNOS activity is induced. Generally, however, these two cNOS isoforms produce small amounts of NO over a period of a few minutes (189). In contrast, the third isoform is an inducible NOS (iNOS) which can, in activated macrophages, produce large amounts of NO for at least 5 days if L-arginine is replenished (190). iNOS is present in many cell types including macrophages, hepatocytes, fibroblasts, smooth muscle cells, astrocytes and epithelial cells but only after these cells are activated by immunological stimuli, such as bacterial endotoxins or cytokines. iNOS also differs from cNOS isoforms in its activity

which in intact cells is independent of Ca^{2+} transients. At the low level of Ca^{2+} found in resting cells, purified murine iNOS can bind calmodulin so tightly that even the denaturing conditions routinely used for SDS-PAGE do not completely separate calmodulin from iNOS (191). This strong binding allows iNOS to be active without the elevation of Ca^{2+} that is usually required for calmodulin binding and activation of target enzymes. Therefore iNOS is regulated differently from cNOS isoforms.

Protein synthesis inhibitors prevent the enhancement of iNOS activity by either LPS or cytokines, indicating that the induction of NO production depends on the *de novo* synthesis of the enzyme, iNOS (192). In IFN-γ and LPS-stimulated murine macrophages, the induction of NOS activity depends upon the activation of PKC, tyrosine kinase, and endogenous ADP ribosylation factors (193). Furthermore, the induction of iNOS by IFN-γ and LPS stimulation is completely blocked by actinomycin D, an inhibitor of RNA synthesis, which demonstrates that iNOS is induced at the transcriptional level (184). The iNOS promoter contains the consensus sequences for binding of the transcriptional factors which are involved in the induction by LPS, such as binding sites for NF-κB, AP-1, IFN-γ response element, TNF response element, IFN-stimulated response element and IFN regulatory factor binding element (189). The iNOS promoter can be induced by LPS alone or by IFN-γ + LPS. IFN-γ synergizes with LPS to enhance the transcription rate of iNOS (192) and to stabilize iNOS mRNA (194), so that the accumulation of iNOS mRNA increases.

As described, the initiation of NO production in macrophages requires IFN-γ and LPS stimulation. While LPS is a potent trigger signal for NO synthesis in IFN-γ primed macrophages, it is not unique. Resident peritoneal macrophages treated with IFN-γ and exposed to *L. major* amastigotes produced high levels of NO_2^-, which is an end product of the reaction between NO with oxygen and water (195), and killed the parasite without

additional LPS (196). Thus, the amastigotes themselves provided the signal for the initiation of NO production in IFN-γ primed macrophages, triggering their own destruction (196). Other effective trigger signals for IFN-γ-primed macrophages include the promastigote form of *L. major*, *T. gondii*, the yeast *Saccharomyces cerevisiae*, and the bacterium *Francisella tularensis* (197).

An important cytokine involved in the regulation of NO production is TNF-α. TNF-α produced by activated macrophages acts in an autocrine fashion to enhance the expression of iNOS mRNA (198, 199). In parasitic infections, endogenous TNF-α plays an essential role in the induction of NO production and in the killing of *L. major* (196), *E. histolytica* (199) and *T. gondii* (200). However, as with TNF-α, high output of NO by macrophages can contribute to host autotoxicity, as NO plays a role in hypotension, destruction of pancreatic islet β cells, arthritis and the depression of local T cell proliferation (201). The characteristics of NO, its high chemical reactivity and its ability to freely diffuse across cell membranes without requirement for a transport mechanism, suggest that the specificity of its interactions are minimal. Therefore, not only is the induction of iNOS an important regulatory step, but its termination must also be rigidly controlled. Macrophages can inhibit NO production through the release of arginase, an enzyme which depletes the substrate, extracellular L-arginine (202) and/or can also release reactive oxygen intermediates (ROI) which scavenge nitrogen oxides (180, 203). Finally, activated macrophages and other immune cells produce regulatory cytokines, such as TGF-β (204), IL-10 (205) and IL-4 (206, 207), which suppress NO production.

In summary, activated macrophages produce RNI as a defensive weapon. TNF-α, produced by these macrophages, acts in a autocrine fashion to enhance NO production through the induction of iNOS mRNA, which is the enzyme responsible for the synthesis

of NO. NO is cytotoxic for tumor cells and many pathogens such as bacteria, yeasts, and helminthic and protozoan parasites.

3.4 References

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SECTION II: MANUSCRIPTS

MANUSCRIPT I

Entamoeba histolytica stimulates the unstable transcription of c-*fos* and tumor necrosis factor-α mRNA by protein kinase C signal transduction in macrophages*

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ABSTRACT

Macrophages play an important role in the control of and resistance to *Entamoeba histolytica*. However, *E. histolytica* infections are characterized by suppression of cell-mediated immunity. To elucidate the molecular mechanisms whereby amebae modulate host defences, we investigated whether the parasite elicits the "immediate early" gene c-*fos* and cytokine TNF-α mRNA and determined the signal transduction pathways involved in naive bone marrow-derived macrophages (BMM). *E. histolytica* stimulated a rapid and transient expression of c-*fos* and low levels of TNF-α mRNA, whereas the nonpathogenic *E. moshkovskii* did not. Inhibition of the protein kinase C (PKC) pathway with the pharmacological inhibitor H7 and by PKC depletion with phorbol myristate acetate showed that *E. histolytica* modulates TNF-α and c-*fos* gene expression through a PKC-dependent stimulus-response coupling event. *E. histolytica* activated and translocated PKC to the membrane fraction in BMM demonstrating a rapid and direct effect on PKC enzyme activity. Unlike LPS, BMM stimulated with *E. histolytica* had reduced stability of both c-*fos* and TNF-α mRNA transcripts (> 50%) and failed to secrete TNF-α protein. BMM treated with amebic proteins and stimulated with LPS, or IFN-γ + LPS, resulted in a 33% and 50% reduction in TNF-α mRNA levels, respectively. These data suggest that although *E. histolytica* stimulates c-*fos* and TNF-α gene expression through PKC signal transduction, the rapid degradation of the mRNAs, the lack of secreted TNF-α protein and the observed decreased responsiveness to a stimulatory signal may be a novel mechanism whereby the parasite modulates host defence mechanisms.

INTRODUCTION

Entamoeba histolytica infects an estimated 10% of the world's population and, with 50,000 to 100,000 deaths annually, it is surpassed only by malaria and schistosomiasis as the leading parasitic cause of death (1). While humoral immunity is not protective (2), cell-mediated immune responses have been shown to be important in the control of and resistance to reinfection (3-7). In clinical studies (6), lymphocytes obtained from patients cured of amebic liver abscess produced, in response to amebic proteins, lymphokines including interferon-gamma (IFN-γ) which activated human monocyte-derived macrophages to kill *E. histolytica* trophozoites. Similarly, spleen and peritoneal macrophages isolated from infected or immunized animals demonstrated increased amebicidal activity (8). *In vitro* studies have shown that murine bone marrow-derived macrophages (BMM) activated with IFN-γ, tumour necrosis factor-alpha (TNF-α) or colony stimulating factor-1, or combinations thereof, exhibited potent amebicidal effects in a contact-dependent manner (3). Amebic killing was not the direct result of IFN-γ or TNF-α cytotoxicity towards the parasite, but rather was accomplished through the activation of macrophages to release the cytotoxic molecule nitric oxide (4, 5), cytolytic proteases and reactive oxygen intermediates (H_2O_2, O_2^-) (3). Recently, we have shown that TNF-α produced by activated macrophages is necessary for enhanced expression of the inducible nitric oxide synthase mRNA in macrophages for nitric oxide-dependent cytotoxicity against *E. histolytica* trophozoites (5). Therefore, TNF-α produced by macrophages is central for cell-mediated immunity against amebiasis.

Although activated macrophages are amebicidal, macrophages isolated from amebic liver granulomas are not effective in controlling the spread of the parasite as amebic liver abscesses grow unrelentingly in immunocompetent hosts, suggesting that macrophage functions are suppressed *in vivo*. Macrophages isolated from amebic liver

granulomas are defective for the production of TNF-α (9), nitric oxide (7), H_2O_2 (10), IL-1 (10) and Ia antigen expression (11), and are refractory to IFN-γ + lipopolysaccharide (LPS) activation for killing of amebae or P815 tumor targets (sensitive to NO) (7). Similarly, elicited murine peritoneal macrophages exposed to soluble amebic proteins *in vitro* show diminished release of H_2O_2 and IL-1 in response to LPS stimulation (10). Naive murine macrophages exposed to soluble amebic proteins *in vitro* produce 70% less TNF-α in response to LPS stimulation than macrophages without amebic exposure (9). Interestingly, peritoneal and splenic macrophages isolated from infected animals are not defective bu. are in a heightened state of activation (9), suggesting that immunomodulation is a local event associated with macrophages in close proximity to the parasites. Taken together, these data suggest that although activated macrophages are potent for amebic killing, amebic components can modulate macrophage effector and accessory cell functions. Amebic liver granuloma macrophages at the margin of the lesions are continually exposed to excretory and secretory amebic components as well as whole cell lysates *in vivo*, thus there is ample opportunity for amebic molecules to modulate macrophage functions.

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, a process commonly described as signal transduction. Two of the better characterized signalling pathways are those utilizing protein kinase C (PKC) and PKA. PKC stimulation has been closely linked with macrophage activation (12, 13) while PKA activation by cAMP analogues has been associated with decreased TNF-α mRNA accumulation in macrophages (14). The c-*fos* gene is among the immediate early genes that are expressed immediately after macrophage activation with LPS and represents a useful nuclear marker to examine signal transduction in macrophages (12). PKC stimulation of c-*fos* mRNA in BMM is rapid and transient, while PKA stimulation of

c-*fos* gene expression via cAMP addition is stable for many hours (15, 16). This difference in kinetics is one means of distinguishing between the pathways.

In the present study, we determine the molecular and biochemical mechanisms whereby *E. histolytica* modulates host immune responses by evaluating gene expression of c-*fos* and TNF-α mRNA and the signal transduction pathways involved in naive BMM exposed to soluble amebic components. Our results demonstrate that BMM stimuiated with *E. histolytica* proteins cause the induction of c-*fos* and TNF-α mRNA expression via the PKC signal transduction pathway. In addition, since both TNF-α and c-*fos* mRNA induced by *E. histolytica* was rapidly degraded and no TNF-α protein was secreted, this suggests a novel mechanism whereby the parasite can suppress macrophage function.

MATERIALS AND METHODS

Reagents. RPMI 1640 medium with L-glutamine was purchased from GIBCO/BRL Life Technologies (Burlington, Ontario, Canada) and was supplemented with 100 U of penicillin per ml, 100 µg of streptomycin sulfate per ml, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) (complete medium). Recombinant TNF-α, rabbit anti-mouse TNF-α polyclonal antibody and hamster anti-mouse TNF-α monoclonal antibody were purchased from Genzyme (Cambridge, Mass.). LPS (*Escherichia coli* serotype 0111:B4, phenol extract) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis, Mo.). The PKC inhibitor, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine hydrochloride (H7), was purchased from Calbiochem Corporation (La Jolla, Ca.). Actinomycin D was obtained from GIBCO/BRL Life Technologies. For the mixed micelle assay to measure PKC activity, ^{32}P-gamma ATP was obtained from Amersham Canada Ltd. (Oakville, Ontario) and [Ser^{25}PKC]

substrate peptide from GIBCO/BRL Life Technologies.

Preparation of bone marrow-derived macrophages. Bone marrow-derived macrophages (BMM) isolated from punctured femurs and tibia of 42 day old female BALB/c mice (Charles River, Canada) were incubated at 37°C in complete RPMI medium overnight on adherent petri dishes (Becton Dickinson, Lincoln Park, N.J.) in the presence of 20% (vol/vol) L929-conditioned medium (source for colony-stimulating factor-1). The following day, non-adherent cells were transferred to non-adherent plates (Baxter Diagnostics Corporation, Mississauga, Ontario, Canada) and allowed to differentiate for 6 days in the presence of 15% (vol/vol) L929-conditioned medium in complete RPMI 1640 medium. Macrophages were made quiescent by washing with Dulbecco's phosphate-buffered saline (PBS, pH 7.2, GIBCO/BRL) and cultured (1 X 10^6 cells/ml) for 12-18 hr in CSF-1-free complete RPMI medium prior to being used.

Preparation of soluble amebic proteins. *E. histolytica* (strain HM1-IMSS) originally provided by L. Diamond (National Institutes of Health, Bethesda, Md.) was cultured in our laboratory as previously described (3). *E. moshkovskii* (Laredo stain), a non-pathogenic ameba, was used as a control (day 10 culture at 22°C). *E. histolytica* trophozoites in mid-log phase (3 days) were used in all studies. Briefly, trophozoites were collected by centrifugation (300 X *g*, 5 min, 4°C) and washed twice with endotoxin-free Dulbecco's PBS (pH 7.2). The trophozoites were placed at -120°C for 10 min and warmed to 37°C to induce a gentle freeze-thaw lysis of the cells. The lysed cells were centrifuged (15,000 X *g*, 5 min, 4°C) to remove cellular debris and the supernatant containing the soluble amebic proteins was collected. The protein concentration was quantified by the method of Bradford (17) (Bio-Rad Laboratories, Mississauga, Ontario, Canada) using bovine serum albumin as a standard. Amebic proteins were free of endotoxin as measured by the E-TOXATE assay (sensitive to 0.05-0.5 endotoxin units per

ml, Sigma). BMM were incubated with 50 µg/ml of freshly prepared *E. histolytica* or *E. moshkovskii* proteins in all studies.

RNA isolation and Northern blot analysis. Total RNA from BMM was isolated by RNAzol (Biotecx International, Friendswood, Tx.), a variation of the guanidinium thiocyanate-phenol-chloroform method (18). Briefly, BMM were collected by centrifugation and lysed with RNAzol. Chloroform was added and the cells were centrifuged at 12,000 X *g* for 15 min at 4°C. The aqueous phase was removed and an equal amount of isopropanol was added to precipitate the RNA. RNA was centrifuged and washed with cold 75% ethanol and resuspended in 1 mM EDTA. The RNA concentration was determined by optical density at 260/280 nm. RNA samples (10 µg) were denatured for 1 hr at 50°C in the presence of glyoxal prior to electrophoresis in a 1% agarose gel in 10 mM sodium phosphate buffer. RNA was blotted by capillary transfer to a Hybond-N nylon membrane (Amersham, Oakville, Ontario, Canada), then UV cross-linked to immobilize the RNA. The membrane was prehybridized for 3 hr at 42°C in a solution containing 5 X SSPE, 50 X Denhardt's solution (1% Ficoll, 1% polyvinvyl-pyrrolidone and 1% BSA), 50% formamide, 0.25 mg/ml of salmon sperm DNA and 0.1% SDS. The cDNA probes were labelled using nick translation (Amersham, Oakville, Ontario, Canada) to incorporate 125 µCi of ^{32}P (ICN Biomedicals, Mississauga, Ontario, Canada) into the cDNA with any excess label removed through a spun column of Sephadex G-50. Labelled probes were incubated with the membrane for 12-18 hr at 42°C. The membrane was washed once with 0.5 X SSC at room temperature, then twice with 0.5 X SSC at 42°C, and again washed with 0.1 X SSC at 65°C for the actin probe. The membrane was autoradiographed using Kodak XAR-5 film with an intensifying screen for 12-18 hr at -70°C. Optical density of the bands was analysed to determine the concentration of probe bound to the membrane. The c-*fos* probe consisted of a 1.3 kb *Pvu*II-*Bgl*II fragment from pFBH-1 from N. Teich, ICRF London, England. The TNF-α

probe was the 1.5 kb *Pst*I fragment from pmTNF-1 from W. Fiers, University of Ghent, Belgium. The actin probe consisted of the 1.25 kb *Pst*I fragment of pBA-1.

TNF-α ELISA. TNF-α production was measured by an ELISA method as described elsewhere (5, 19). TNF-α concentration was measured by using recombinant mouse TNF-α as a standard. Data are expressed as U/ml/10^6 cells.

Protein kinase C assay. BMM were cultured as described and incubated for 15 min with either 50 µg/ml *E. histolytica* proteins, 100 ng/ml LPS or 10 µM PMA. PMA was used as a control for PKC activation (20). Cells were collected by centrifugation (1,000 X *g*, 8 min, 4°C) then the membrane and cytosol fractions were isolated. Briefly, cells were snap frozen in liquid N_2, then homogenized in 5 ml of ice cold buffer A (pH 7.4) containing 20 mM HEPES, 20 mM $MgCl_2$, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, pepstatin (2 µg/ml), leupeptin (2 µg/ml), aprotenin (1 µg/ml) and benzamidine (400 µg/ml). High speed supernatants (100,000 X *g* for 60 min at 4°C) containing the cellular cytosol fractions were applied to DE52 columns preequilibrated with buffer B (pH 7.4) containing 20 mM HEPES, 2 mM EGTA, 2 mM EDTA and 2 mM DTT as described (21). The membrane pellet was solubilized with buffer A with 1% (wt/vol) Nonidet-P40, kept on ice for 30 min, recentrifuged (15,000 X *g* for 30 min, 4°C) and the supernatants were added to DE52 columns. After removal of unbound proteins by washing columns with buffer B, fractions containing PKC were eluted with buffer B containing 0.1 M NaCl. The PKC activity in the eluates was determined by the mixed micelle assay (22). The mixed micelle reaction measured the incorporation of ^{32}P-gamma ATP (Amersham) into [Ser^{25}PKC] substrate peptide (GIBCO/BRL). Briefly, reaction mixtures (1 mg/ml DTT, 34 mM NaCl, 11 mM $MgCl_2$, 30 mM HEPES, 700 µM EGTA, 700 µM EDTA, 3 mM $CaCl_2$ and 320 µM [Ser ^{25}PKC]) containing 60 µg/ml of phosphatidylserine and 5 µg/ml of diolein in 0.3% Triton-X (+) and reaction mixtures

without phosphatidylserine and diolein (-) were added to samples of cytosol and membrane fractions. Samples were incubated for 10 min at 30°C and then spotted onto P81 paper. The paper was washed with 1% phosphoric acid and then the activity was determined by scintillation counting. The difference between the activities of the (+) and (-) samples per µl was then divided by the protein concentration of the sample then by the specific activity of the ATP used in the assay. The protein concentrations in cytosol and membrane fractions were quantified by the Bradford protein assay (17).

Statistical analysis. All experiments were performed in triplicate and the results are presented as means $\pm$ S.D. Data were analyzed by the Student's *t* test or by linear regression analysis. A value of $P < 0.05$ is regarded as statistically significant.

RESULTS

***E. histolytica* stimulates c-*fos* and TNF-α gene transcription.** The cytokine TNF-α, produced by activated macrophages, has been shown to be central in stimulating inducible nitric oxide synthase mRNA for nitric oxide-dependent cytotoxicity against *E. histolytica* trophozoites (4, 5). We hypothesized that TNF-α would be modulated by *E. histolytica* so as to enhance parasite survival within tissues and to evade the host immune response. We were interested, therefore, in characterizing the early responses of naive macrophages to *E. histolytica.* As c-*fos* is one of the earliest genes expressed during the activation of macrophages, (12, 13) we included it in our study as a reporter gene. LPS served as a positive control in these experiments as the addition of LPS triggers the accumulation of c-*fos* and TNF-α mRNA (23).

In the first series of experiments, we assessed the kinetics of c-*fos* and TNF-α mRNA expression by Northern analysis in BMM (1×10^6 cells/ml) stimulated with 100 ng/ml of LPS, 50 µg/ml of *E. histolytica* proteins or 50 µg/ml of the nonpathogenic

E. moshkovskii (Laredo) proteins after 0.5, 1 and 3 hr. Quiescent BMM were used to avoid the high basal levels of c-*fos* and TNF-α gene expression present in primed or activated macrophages. As shown in Figure 1, as early as 0.5 hr following stimulation with LPS, c-*fos* mRNA levels increased 5-fold and then rapidly declined to a barely detectable level by 3 hr. Macrophages stimulated with *E. histolytica* proteins caused the induction of c-*fos* gene expression in a manner similar to that of LPS, with peak expression at 0.5 hr following stimulation (4-fold increase) which declined to basal levels by 3 hr. In this study, 50 μg/ml of amebic protein was chosen as the optimal concentration as determined by a dose response (1.0, 10, 25, 50 and 100 μg/ml) experiment which demonstrated maximal c-*fos* and cytokine gene expression with this concentration of protein (data not shown). Interestingly, equivalent amounts of the nonpathogenic *E. moshkovskii* protein also stimulated c-*fos* gene expression in a similar fashion as *E. histolytica* and LPS. However, *E. moshkovskii* was unable to stimulate TNF-α mRNA expression, whereas *E. histolytica* stimulated TNF-α mRNA levels 3-fold at 0.5 hr, which remained stable up to 3 hr (Fig. 1). In response to LPS stimulation, TNF-α mRNA levels increased 3-fold after 0.5 hr, and 11 and 13-fold after 1 and 3 hr, respectively. Therefore, although the pattern of c-*fos* mRNA expression was similar for all three stimuli, the pattern of TNF-α gene expression differed between LPS and *E. histolytica* stimulation and was nonexistent for *E. moshkovskii* stimulation.

***E. histolytica* stimulates c-*fos* and TNF-α through PKC signal transduction.** Macrophage activation has been closely linked to PKC signal transduction, (12, 13) while another pathway, PKA, has been associated with decreased TNF-α mRNA accumulation (14). LPS and other activators of the PKC pathway induce a rapid (within 0.5 hr) and transient expression of c-*fos* mRNA, while PKA activators stimulate stable c-*fos* mRNA expression for many hours (15, 16). We were interested, therefore, in determining which

signal transduction pathway *E. histolytica* utilizes to modulate macrophage functions and used c-*fos* as a reporter gene for this purpose.

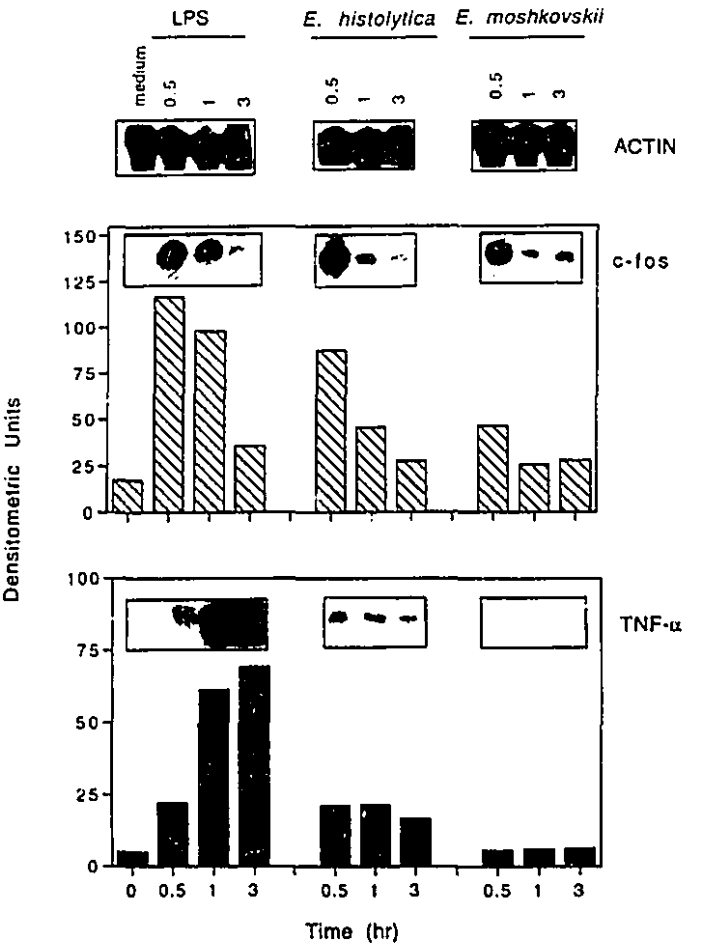

Figure 1. c-*fos* and TNF-α mRNA expression in BMM exposed to LPS and a: ebae. BMM were stimulated with LPS (100 ng/ml), *E. histolytica* (50 μg/ml) or *E. moshkovskii* proteins (50 μg/ml) for 0.5, 1.0 and 3 hr. Total RNA was isolated and the levels of c-*fos*, TNF-α and actin mRNAs were assessed by Northern blot analysis as described. Results of scanning densitometric analysis of Northern blots are presented as a histogram after c-*fos* or TNF-α levels were standardized relative to actin levels and expressed as densitometric units. Lane designations are identical for both blot and histogram.

To determine whether the pattern of c-*fos* mRNA expression in response to *E. histolytica* occurred through a PKC signal transduction event, two methods were chosen to inhibit PKC prior to LPS or *E. histolytica* exposure. First, BMM were pretreated with the PKC inhibitor, H7 (20 μM; PKC IC_{50}=6 μM, PKA IC_{50}=3 μM), for 5

minutes and then stimulated with LPS or *E. histolytica*. As shown in Figure 2, c-*fos* mRNA levels rapidly increased within 0.5 hr following both LPS and *E. histolytica* stimulation and fell to low levels within 3 hr. This rapid and transient increase in c-*fos* mRNA expression is characteristic of a PKC signal transduction event. In H7-pretreated cells, c-*fos* mRNA expression was inhibited by 50% in response to both stimuli at 0.5 and 1.0 hr; at 3 hr c-*fos* mRNA expression was abrogated. With regard to TNF-α mRNA expression, in response to LPS stimulation, there was a modest increase in TNF-α mRNA after 0.5 hr which increased rapidly thereafter to a 25-fold increase after 3 hr (Fig. 2).

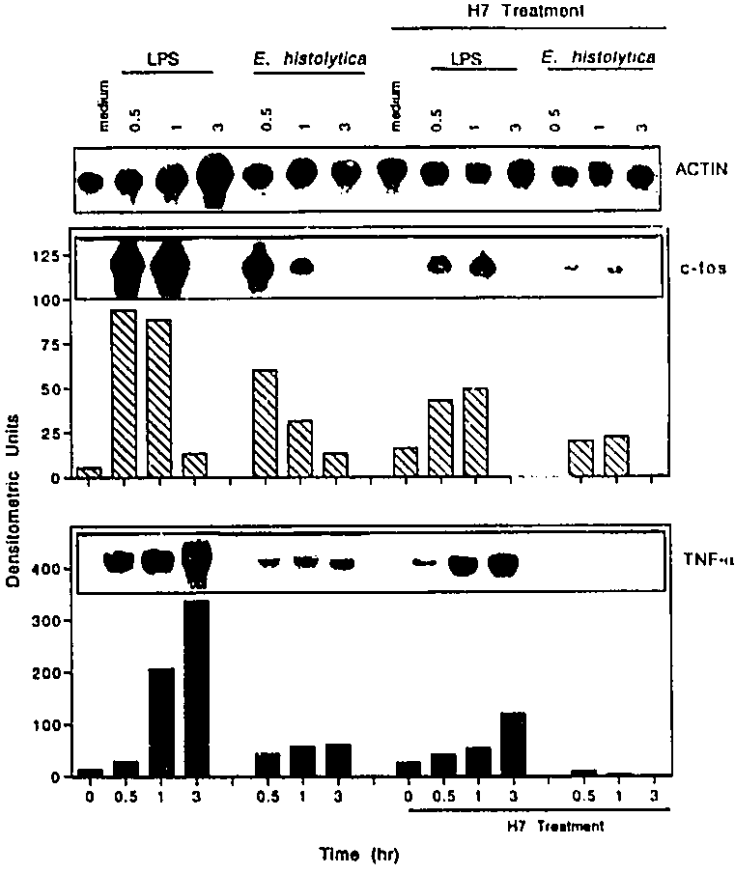

Figure 2. Effect of PKC inhibition with H7 pretreatment on c-*fos* and TNF-α mRNA expression in BMM exposed to LPS and *E. histolytica*. BMM were pretreated with H7 (20 μM) for 5 min and then stimulated with LPS (100 ng/ml) or *E. histolytica* proteins (50 μg/ml) for 0.5, 1 and 3 hr. Total RNA was isolated and the levels of c-*fos*, TNF-α and actin mRNAs were assessed by Northern blot analysis as described. Results of scanning densitometric analysis of Northern blots are presented as a histogram after c-*fos* and TNF-α levels were standardized relative to actin levels and expressed as densitometric units. Lane designations are identical for blots and histograms.

E. histolytica proteins stimulated a modest increase in TNF-α mRNA expression (Fig. 2), but in a pattern different from that of LPS. *E. histolytica*-stimulated TNF-α mRNA levels were increased 4-fold over control levels as early as 0.5 hr following exposure and were maintained at the same level for the next 3 hr. Despite this difference in the pattern of TNF-α mRNA induction, H7 pretreatment inhibited both LPS and *E. histolytica*-induced TNF-α mRNA by 60 and 100%, respectively, after 3 hr (Fig. 2). Lower concentrations of H7 (< 10 μM) failed to reduce c-*fos* or TNF-α mRNA levels in response to either LPS or *E. histolytica* stimulation.

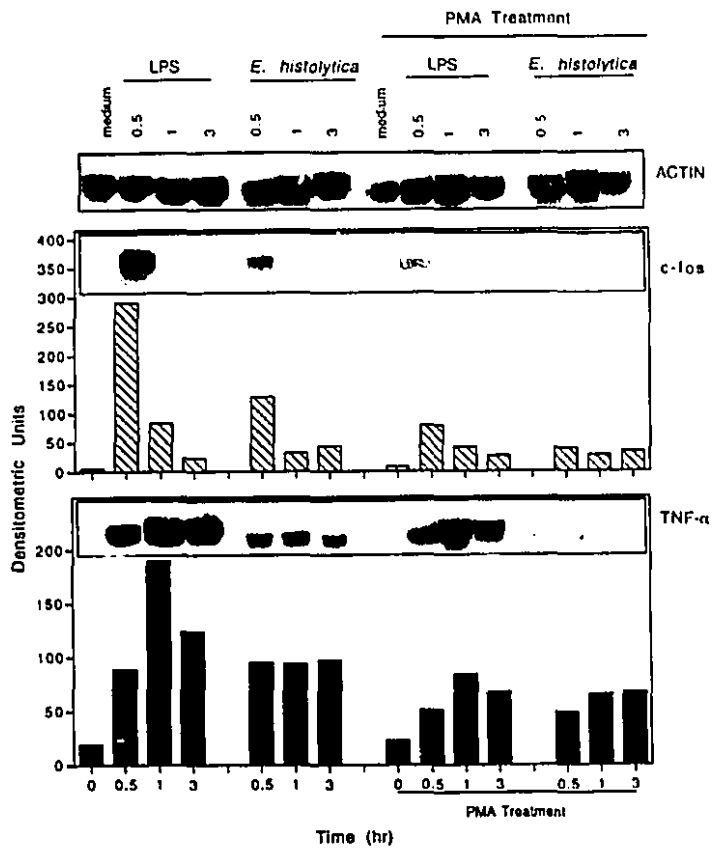

Figure 3. Effect of PKC depletion by PMA stimulation on c-*fos* and TNF-α mRNA expression in BMM exposed to LPS and *E. histolytica*. BMM were pretreated with PMA (10 μM) for 18 hr and then stimulated with LPS (100 ng/ml) or *E. histolytica* proteins (50 μg/ml) for 0.5, 1 and 3 hr. Total RNA was isolated and the levels of c-*fos*, TNF-α and actin mRNAs were assessed by Northern blot analysis as described. Results of scanning densitometric analysis of Northern blots are presented as a histogram after c-*fos* and TNF-α levels were standardized relative to actin levels and expressed as densitometric units. Lane designations are identical for blots and histogram.

A second method chosen to inhibit PKC was by the addition of PMA for 18 hr prior to stimulation with LPS or amebic proteins. Prolonged PMA exposure exhausts the supply of PKC in macrophages (24). PMA pretreatment reduced c-*fos* mRNA expression to both LPS and *E. histolytica* by 75% as shown in Figure 3. In PKC-deficient cells, TNF-α mRNA expression was inhibited by 63% and 35% in response to LPS and *E. histolytica*, respectively, after 3 hr. These results indicate that *E. histolytica* stimulates c-*fos* and TNF-α mRNA expression in BMM partially through PKC signal transduction and that *E. histolytica* components can directly modulate macrophage functions.

***E. histolytica* activates and translocates PKC in macrophages.** As with any pharmacologic inhibitor, we could not rule out that the alteration of biochemical pathways other than PKC may have contributed to the effect of H7 (15). Even though the molarity of the inhibitor used was specific for PKC and lower concentrations (1, 5 10 μM) had no effect, to be certain that PKC signal transduction events were occurring, we measured PKC specific activity in the membrane and cytosol fractions of BMM exposed briefly to LPS (100 ng), PMA (10 μM) or *E. histolytica* proteins (50 μg). As shown in Figure 4, in control unstimulated BMM, there were low basal amounts of PKC activity in the cytosol or membrane fractions. However, in BMM stimulated with either LPS or PMA for 15 min, there was a 4-fold increase in PKC activity in the membrane fraction as compared to the respective cytosol fractions indicating translocation of the active enzyme ($P < 0.05$). Similarly, in BMM stimulated with *E. histolytica* for 15 or 30 min, there was a 2-fold increase in PKC activity in the membrane fraction as compared to the cytosol fraction ($P < 0.05$), indicating PKC activation and translocation of the enzyme from the cytosol to the membrane. These results, coupled with the fact that PKC inhibitors can alter cytokine and c-*fos* gene expression in response to *E. histolytica*, clearly indicate that the parasite can modulate signal transduction events via PKC in BMM.

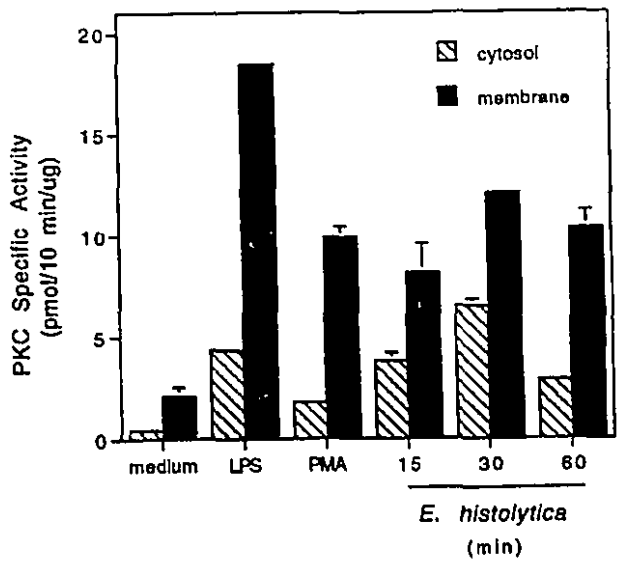

Figure 4. PKC specific activity in the membrane and cytosol fractions of BMM exposed to various stimuli. BMM were stimulated with LPS (100 ng/ml) or PMA (10 μM) for 15 min or *E. histolytica* proteins (50 μg/ml) for 15, 30 and 60 min prior to measuring PKC activity in the membrane and cytosol fractions of the cells by a PKC assay as described. $P<0.05$ for PKC specific activity in the membrane as compared to the cytosol fractions for each treatment.

***E. histolytica*-stimulated TNF-α and c-*fos* gene transcripts have reduced stability.** The steady state levels of TNF-α mRNA in macrophages exposed to *E. histolytica* appear to correlate with the previously observed constitutive low basal levels of TNF-α production by amebic liver abscess-derived macrophages (9). However, this correlation was not verified as there was no evidence of TNF-α protein in the medium of BMM stimulated for 3 hr with amebic proteins as measured by TNF-α ELISA, whereas in response to 3 hr of LPS stimulation BMM produced 111 ± 13 U of TNF-α/10^6 macrophages ($P < 0.05$). This indicates, that the low levels of TNF-α mRNA expressed in BMM stimulated with *E. histolytica* were not translated into protein. Accordingly, we determined the stability of the TNF-α mRNA transcripts expressed in response to amebic stimulation by performing an actinomycin D (to inhibit transcription) stability experiment, using the stability of TNF-α mRNA transcripts expressed in response

to LPS as a standard. As shown in Figure 5, macrophages stimulated for 3 hr with LPS expressed increased levels of TNF-α mRNA that degraded slowly with time returning to undetectable levels after 6 hr. In contrast, in macrophages stimulated with *E. histolytica*, TNF-α mRNA degraded precipitously and was undetectable after 1 hr. The stability of the TNF-α mRNA as determined by the half life of the % RNA remaining ($t_{½}$=50% mark on linear slope) in response to *E. histolytica* was significantly reduced ($P<0.05$) as compared to that observed in response to LPS stimulation (< 0.5 hr vs > 2.0 hr; Fig. 5).

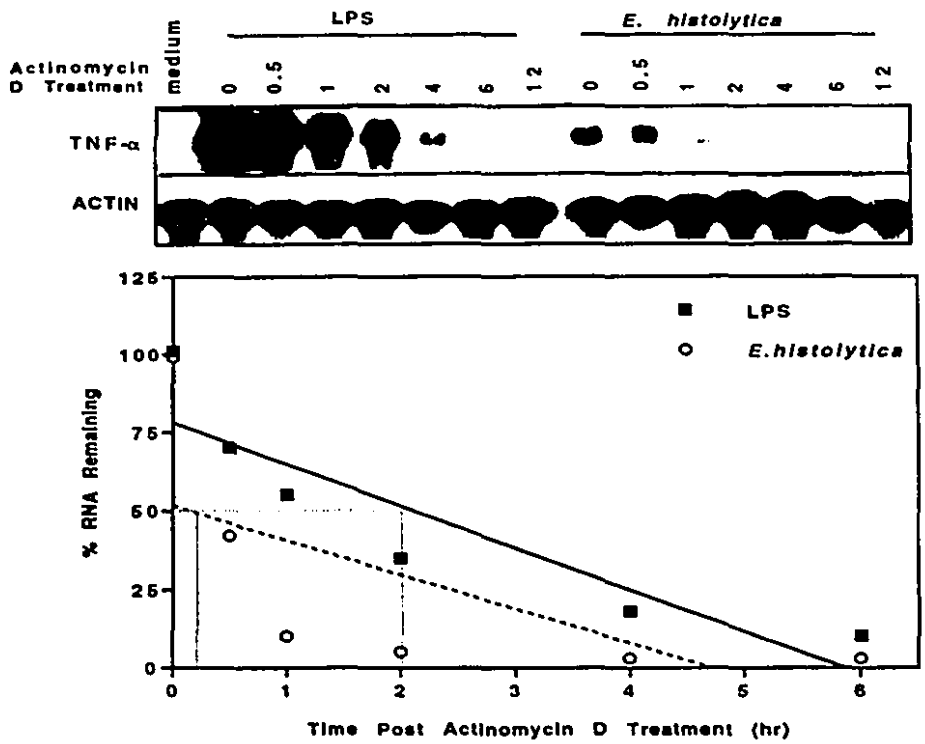

Figure 5. Stability of TNF-α mRNA in BMM. BMM were stimulated with either LPS (100 ng/ml) or *E. histolytica* proteins (50 μg/ml) for 3 hr and then treated with actinomycin D (10 μg/ml). Total RNA was isolated at various times following actinomycin D treatment and Northern blots were performed. TNF-α and actin levels were calculated by densitometry of autoradiographs. TNF-α mRNA levels are expressed as a percentage of the mRNA level determined before the addition of actinomycin D (3 hr post-stimulation mRNA levels=100% RNA remaining in graph). The $t_{½}$ (50% stability) was calculated for both LPS (solid line, r=0.903) and *E. histolytica*-stimulated (broken line, r=0.661) BMM.

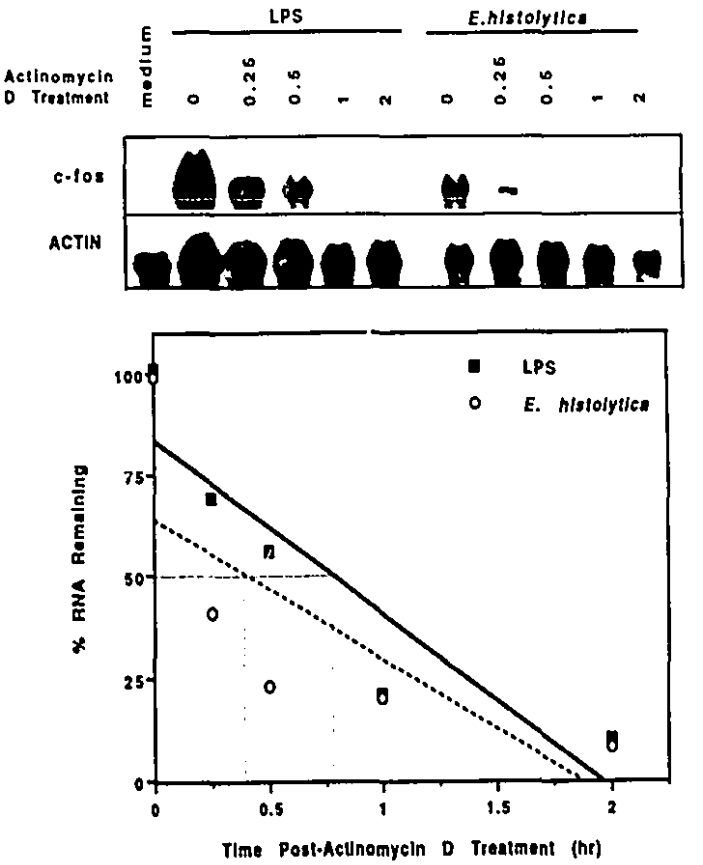

Figure 6. Stability of c-*fos* mRNA in BMM. BMM were stimulated with either LPS (100 ng/ml) or *E. histolytica* proteins (50 µg/ml) for 0.5 hr and then treated with actinomycin D. Total RNA was isolated at various times following actinomycin D treatment and Northern blots were performed as described. Densitometry of autoradiographs for both c-*fos* and actin levels were performed. c-*fos* mRNA levels are expressed as a percentage of the mRNA level determined before the addition of actinomycin D (0.5 hr post-stimulation mRNA levels=100% RNA remaining in graph). The $t_{1/2}$ (50% stability) was calculated for both LPS (solid line, r=0.914) and *E. histolytica*-stimulated (broken line, r=0.681) BMM.

In a parallel experiment, we also measured the stability of c-*fos* mRNA as the c-*fos* gene product is involved in the regulation of other genes (13). In macrophages stimulated with LPS for 0.5 hr (time of maximal c-*fos* expression), c-*fos* mRNA degradation occurred at a rate such that following 1 hr after the addition of actinomycin D, the levels of c-*fos* mRNA were undetectable (Fig. 6). In contrast, in *E. histolytica*-exposed cells (0.5 hr stimulation), c-*fos* mRNA degraded rapidly within 0.25

hr and was undetectable after 0.5 hr (Fig. 6). In LPS-treated and *E. histolytica*-treated cells, the $t_{1/2}$ for c-*fos* mRNA was > 0.75 hr and < 0.50 hr, respectively ($P<0.05$, Fig. 6). These data demonstrate that even though *E. histolytica* proteins stimulated TNF-α and c-*fos* mRNA expression, the mRNA was rapidly degraded with an approximate 50% reduction in stability.

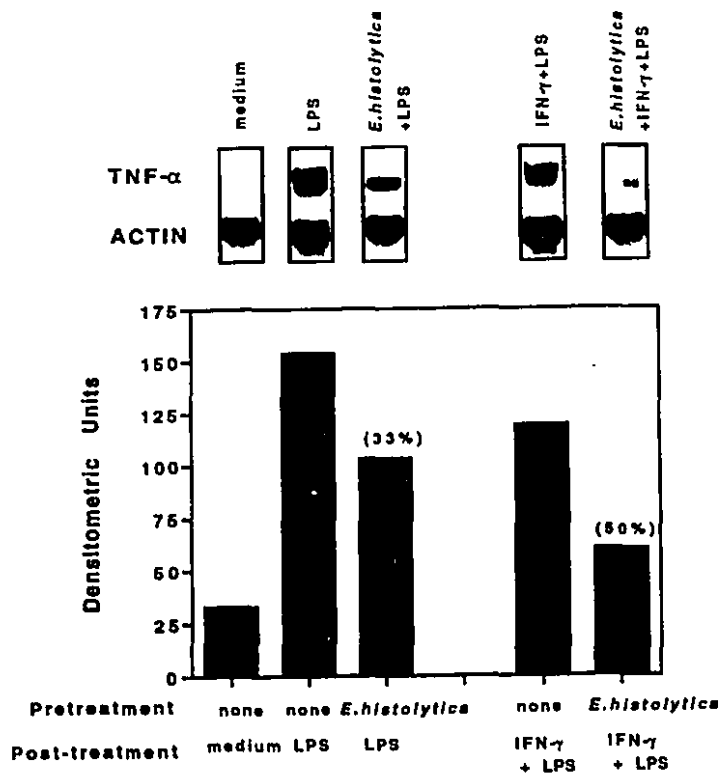

Figure 7. Effect of *E. histolytica* pretreatment on LPS-stimulated TNF-α mRNA expression. BMM were treated with amebic proteins (50 μg/ml) for 3 hr then stimulated with LPS (100 ng/ml) for 3 hr or with IFN-γ (100 Units/ml) + LPS for 24 hr. Total RNA was isolated and the levels of TNF-α and actin mRNAs were assessed by Northern blot analysis as described under "Materials and Methods". Results of scanning densitometric analysis of Northern blot are presented as a histogram after TNF-α levels were standardized relative to actin levels and expressed as densitometric units. Lane designations are identical for both blot and histogram. Values in parentheses are percent inhibition compared to the homologous controls.

Pretreatment of macrophages with *E. histolytica* inhibits TNF-α mRNA expression. The results of the previous experiment demonstrate that *E. histolytica* stimulates the expression of both c-*fos* and TNF-α mRNA in BMM and this mRNA is unstable, which may be a mechanism whereby the parasite suppresses macrophage

functions. In order to determine whether preexposure of BMM with amebic proteins could modulate macrophage responsiveness to a second signal (i.e. immunomodulation), BMM were pretreated with amebic proteins for 3 hr and then stimulated with LPS for 3 hr, or IFN-γ + LPS for 24 hr, and the levels of TNF-α mRNA determined. As shown in Figure 7, BMM pretreated with amebic proteins demonstrated a 33% decrease in TNF-α mRNA levels in response to LPS as compared to LPS treatment alone. In BMM pretreated with amebic proteins and stimulated with IFN-γ + LPS, there was a 50% decrease in TNF-α mRNA expression as compared to macrophages stimulated with IFN-γ + LPS alone (Fig. 7). These findings demonstrate that amebic molecules can suppress, at the mRNA level, the response of naive macrophages to well-characterized activating agents, such as LPS, or IFN-γ + LPS.

DISCUSSION

As amebic components modulate macrophage functions during the course of the infection, the focus of this investigation was to determine the molecular events associated with this phenomenon. Our studies demonstrated that *E. histolytica* had an effect on macrophage functions by stimulating the mRNA expression of the transcription factor, c-*fos*, and the cytokine, TNF-α. In the absence of stimulation, macrophages did not express detectable levels of c-*fos* or TNF-α mRNAs. The rapid and brief expression of c-*fos* in response to both LPS and *E. histolytica* is indicative of a PKC signal transduction event. TNF-α mRNA expression was increased 4-fold in response to *E. histolytica* and was maintained at the same level for up to 3 hr. Inhibition of PKC with the pharmacological inhibitor, H7 (20 μM only), or depletion of PKC by PMA treatment (24) greatly reduced c-*fos* mRNA expression in response to the parasite which demonstrates the involvement of PKC-dependent stimulus-response coupling in BMM exposed to *E. histolytica*. Similarly, abrogation of TNF-α mRNA expression with the PKC inhibitor,

H7, and reduction in TNF-α mRNA levels following PMA pretreatments suggest that TNF-α mRNA expressed in response to *E. histolytica* is also transduced through the PKC pathway.

E. histolytica stimulated the activation and translocation of PKC to the membrane within 15 min of exposure. A requirement for signal transduction is that PKC must not only be stimulated, but also translocated to the plasma membrane. Translocation of PKC to the plasma membrane is stringently regulated and can be induced without PKC activation by increasing intracellular levels of Ca^{2+} (25). The observed effect of *E. histolytica* on macrophages was not solely the result of increased intracellular Ca^{2+} levels, as both PKC translocation and activation occurred as measured by the increased TNF-α and c-*fos* mRNA levels. The purpose of PKC signal transduction is protein phosphorylation which results in macrophage activation. The induction of c-*fos* mRNA expression in macrophages by LPS has been related to the development of functional competence (13). The regulation of c-*fos* expression has a direct effect on the subsequent cascade of genes expressed during macrophage priming/activation through the DNA binding activity of the FOS protein (15). Thus, the pattern of c-*fos* and TNF-α mRNA expression and PKC enzyme translocation and activation in response to amebic stimulation would suggest that the macrophages are being activated. Although *E. histolytica* increases c-*fos* and TNF-α mRNA levels, the mRNA transcripts have reduced stability with $t_{\frac{1}{2}}$ reduced > 50% as compared to the LPS control. In addition, no TNF-α protein was produced, suggesting that macrophages stimulated by *E. histolytica* have not acquired functional competence. The reduced stability of TNF-α and c-*fos* mRNA may be a novel mechanism invoked by the parasite to suppress macrophage activation. The rate of mRNA decay represents an important checkpoint in the regulation of gene expression and, eventually, protein production. Various cytokines, including TNF-α and proto-oncogenes, have been described as having AU-rich regions in the 3'

untranslated region of mRNA that is implicated in mRNA stability and rapid turnover of the message (26). Similar to our studies with *E. histolytica*, *Mycobacterium avium intracellulare* (MAI) has been shown to stimulate TNF-α mRNA levels at 1.5 to 2 hr which returned to base line levels after 6 hr (27). The stability of the MAI-induced TNF-α mRNA was greatly reduced as compared to LPS-stimulated gene expression ($t_{½}$=18 min vs $t_{½}$=60 min) in control cells (27). MAI resides in macrophages and most likely uses decreased cytokine mRNA stability as a strategy to prevent long-term and high level production of TNF-α. It is possible that *E. histolytica* exploits a similar response in macrophages to decrease macrophage activation, specifically TNF-α production, which regulates nitric oxide-mediated cytotoxicity against amebae (4, 5) in order to evade the host immune response. Repressor factors may be involved in the diminished mRNA stability expressed in response to *E. histolytica* and will be an interesting area to pursue in future studies.

It has long been recognized that an initial sublethal exposure to LPS renders experimental animals and human subjects refractory to subsequent challenge with LPS, but the precise mechanism of this endotoxin tolerance remained unclear (29). Takasuka *et al.* (28) demonstrated that the LPS-refractory state in TNF-α production occurs at the mRNA level. Although our results demonstrate that, after a brief initial exposure to amebic proteins, naive macrophages express TNF-α and c-*fos* mRNA, our pretreatment experiments demonstrate a reduction in TNF-α mRNA levels, suggesting that continuous exposure to amebic components, which occurs within an amebic liver abscess, may render macrophages refractory to further stimulation by the parasite or known activators, such as LPS, or IFN-γ + LPS. The immunosuppression observed in invasive amebiasis could be analogous to the mechanism of endotoxin tolerance. Specifically, amebic proteins have been shown to suppress LPS-induced TNF-α production (9), IFN-γ-induced Ia antigen expression (11), respiratory burst potential (10) and nitric oxide production (7)

and to increase production of two inflammatory molecules PGE_2 and LTC_4 (30), suggesting that soluble amebic proteins are important modulators of immune cell functions.

In conclusion, our studies clearly demonstrate that *E. histolytica* stimulates PKC activation and translocation, and increased transcription of c-*fos* and the pro-inflammatory cytokine, TNF-α mRNA in naive macrophages. Even though the stimulatory response is similar to macrophage activation, the reduced stability of c-*fos* and TNF-α mRNAs and the absence of any secreted TNF-α protein suggest that the macrophages are not functionally competent. Further evidence is the 33% to 50% reduction in TNF-α mRNA expression in amebic protein-pretreated BMM stimulated with LPS, or IFN-γ + LPS. Therefore, the "false activation" of the macrophages observed in this study appears to be a novel mechanism by which the parasite modulates host defenses.

ACKNOWLEDGMENTS

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CONNECTING STATEMENT I

In the first manuscript, soluble components from *E. histolytica* were shown to stimulate naive macrophages for enhanced TNF-α and c-fos mRNA expression and activate Protein Kinase C signal transduction within the macrophages. However, the mRNA expression was unstable for both genes, and no TNF-α protein production was observed in response to *E. histolytica*. As soluble *E. histolytica* proteins contain many molecules that can stimulate, or inhibit, macrophage functions we were interested in determining what effect a single amebic molecule would have upon macrophage functions. We selected the galactose adherence lectin as it is central for amebic pathogenesis, is an immunodominant molecule and a protective vaccine candidate. The following studies investigated the effect of the Gal-lectin on TNF-α mRNA induction in macrophages, and identified a region of the Gal-lectin responsible for mediating TNF-α mRNA induction.

MANUSCRIPT II

Identification of the Galactose-Adherence Lectin Epitopes of *Entamoeba histolytica* that stimulate Tumor Necrosis Factor-alpha production by macrophages*

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ABSTRACT

The 170 kDa subunit of the galactose adherence lectin (Gal-lectin) of *Entamoeba histolytica* mediates adherence to human colonic mucins and intestinal epithelium as a prerequisite to amebic invasion. The Gal-lectin is an immunodominant molecule and a protective antigen in the gerbil model of amebiasis. Tumor necrosis factor-α (TNF-α) produced by activated macrophages enhances nitric oxide-dependent cytotoxicity for host defense against *E. histolytica*. The purpose of this study was to identify the Gal-lectin epitopes which stimulate TNF-α production by macrophages. Murine bone marrow-derived macrophages (BMM) exposed to Gal-lectin (100-500 ng/ml) stimulated stable expression of TNF-α mRNA (8-fold increase), and TNF-α production similar to that of LPS-stimulated cells (100 ng/ml). Polyclonal anti-lectin serum specifically inhibited TNF-α mRNA induction in response to the Gal-lectin but not to LPS. Anti-lectin monoclonal antibodies (mAb) 8C12, H85 and 1G7, which recognize non-overlapping epitopes of the cysteine rich region of the 170 kDa heavy subunit inhibited both amebic adherence to mammalian cells and Gal-lectin-stimulated TNF-α mRNA expression by BMM, but mAb 7F4 did neither. As these inhibitory mAbs map to amino acids 596-1082 of the 170 kDa Gal-lectin, our results have identified a functional region that mediates amebic adherence and TNF-α mRNA induction in BMM, and thus, this region of the Gal-lectin can be used as a potential subunit vaccine candidate.

INTRODUCTION

The enteric protozoan parasite, *Entamoeba histolytica*, causes the disease amebiasis and despite the availability of effective antiamebic chemotherapy approximately 50 000 to 110 000 deaths occur annually (1). Following drug-cure, protective immunity against invasive amebiasis develops but has no effect on parasite establishment in the colon (2). The reasons for this are unclear and all evidence to date suggests that antibodies are ineffective whereas an effective cell-mediated immune response, mediated by activated macrophages, is important in resistance against reinfections (3-9). Nitric oxide (NO) is the major effector molecule produced by activated macrophages for *in vitro* cytotoxicity against *E. histolytica* trophozoites (10). IFN-γ and TNF-α activate macrophages for cytotoxicity against *E. histolytica* trophozoites *in vitro* (3, 4) by inducing NO production (10). Antiserum to TNF-α suppressed NO production and amebicidal activity as well as TNF-α secretion demonstrating that TNF-α is central for NO production (11).

Studies using partially fractionated or purified amebic proteins (12-17) demonstrated that the production of an effective vaccine against amebiasis may be possible. The major candidate molecule in *E. histolytica* is the Gal-lectin which mediates amebic adherence to human colonic mucins and intestinal epithelium as a prerequisite to invasion (18). The native amebic Gal-lectin is a 260 kDa heterodimer glycoprotein composed of 170 kDa heavy and 31-35 kDa light subunits linked by disulfide bonds (19). The cysteine-rich extracellular domain of the 170 kDa heavy subunit mediates *E. histolytica* adherence to mammalian cells and colonic mucins (20). The 170 kDa heavy subunit is antigenically conserved and is recognized by >90% of human immune sera (21). *In vitro*, the native Gal-lectin stimulates IFN-γ production by T-lymphocytes (22) which is an important cytokine to "prime" macrophages. As a protective antigen to

prevent amebic liver abscess formation in the gerbil model of amebiasis, the native Gal-lectin demonstrated 67% efficacy (12), and a recombinant fusion protein composed of the cysteine-rich region of 170 kDa subunit conferred 71% efficacy when used as a vaccine (23). However, the immunologic basis for the protective immune response elicited by the Gal-lectin is not yet known.

The focus of this study was to determine the cellular and molecular basis whereby the Gal-lectin elicits an effective cell-mediated immune response against *E. histolytica.* Specifically, we sought to identify the functional epitopes of the amebic Gal-lectin 170 kDa subunit which stimulate TNF-α mRNA and protein production by murine BMM as TNF-α is central for NO-dependent cytotoxicity against *E. histolytica* (10, 11). Using a panel of mAbs which recognize non-overlapping epitopes of the cysteine rich extracellular domain of the Gal-lectin (20), we have identified a region containing amino acids (aa) 596-1082 that mediates amebic adherence to target cells and increased TNF-α mRNA levels in BMM.

MATERIALS AND METHODS

Cultivation of E. histolytica and adherence to Chinese Hamster Ovary (CHO) cells. *E. histolytica* (strain HM1-IMSS) was cultured in our laboratory as previously described (3). CHO cells were grown in F12 medium (Gibco/BRL Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 100 U/ml of penicillin and 100 µg/ml of streptomycin sulfate. Amebic adherence to CHO cells was performed as previously described (24). Rosette formation was defined as the percentage of amebae with three or more adherent target cells, which was determined by counting > 100 amebae per tube.

Preparation of BMM, RNA isolation and Northern blot analysis. BMM were isolated from the femurs and tibia of 42 day old female BALB/c mice (Charles River, Canada) as described previously (11). Total RNA from BMM was isolated with Trizol (Gibco) and was electrophoresed in a 1% agarose gel (10 ug RNA/lane). Northern blotting of the membranes was performed for 12-18 h at 42°C using ^{32}P-labelled probes after which the membranes were exposed to Kodak XAR-5 film with an intensifying screen for 12-18 h at -70°C as described (26). Optical density of the bands was analyzed to determine the concentration of probe bound to the membrane. The TNF-α probe was the 1.5 kb *Pst*I fragment from pmTNF-1 from W. Fiers, University of Ghent, Belgium. The actin probe consisted of the 1.25 kb *Pst*I fragment of pBA-1. LPS (*Escherichia coli* serotype 0111:B4, phenol extract, obtained from Sigma, St. Louis, Mo.) was used as a positive control for BMM activation.

Preparation of amebic proteins. Soluble amebic proteins were prepared following freeze-thaw lysis of trophozoites as described (26). The isolation and purification of the native Gal-lectin by mAb affinity chromatography has been described in detail elsewhere (19). In brief, octylglucoside solubilized amebae were applied to a column consisting of purified anti-lectin mAbs H85, 7F4, 5B8, 3F4 and 6D2. Endotoxin contamination was undetectable in 50 µg/ml of soluble amebic proteins or 1 µg/µl of Gal-lectin as measured by the ETOXATE-assay (sensitive to 0.05-0.5 endotoxin units per ml, Sigma).

Production of Gal-lectin mAbs and Western blots. Detailed protocols for the production of murine anti-lectin mAbs have been previously reported (25). A series of successive deletion peptides of the 170 kDa subunit of the Gal-lectin were used to map the regions containing the recognition sites for these mAbs (20). Rabbit polyclonal anti-lectin serum was prepared as described (20). For Western blot analysis, SDS-PAGE of the Gal-lectin (5 µg) and soluble *E. histolytica* proteins (50 µg) was performed with a

10% separating and a 4% stacking gel under reducing conditions. Proteins were transferred to a 45 μM pore size nitrocellulose membrane (Bio-Rad Laboratories) and the membrane was incubated with 3% milk proteins in Tris buffered saline (TBS) overnight at 4°C. Rabbit preimmune serum, immune serum against the Gal-lectin, anti-lectin mAbs (8C12, H85, 1G7, and 7F4), and a control mAb against an irrelevant glycoprotein (R35, IgG1 isotype, recognizes human colonic mucins) were diluted 1:500 in TBS with 1% milk proteins and 0.1% Tween-20. The membrane was incubated with either rabbit anti-mouse Ig horseradish peroxidase conjugated antibody or donkey anti-rabbit Ig horseradish peroxidase conjugated antibody (Amersham Life Science, Oakville, Ontario, Canada) and placed in TBS solution. Enhanced Chemiluminescence (ECL) was performed on the membrane according to the manufacturer's directions (Amersham). The membrane was exposed to Kodak XAR-5 film.

Statistics. All experiments were performed three times, with a single representative experiment shown for Northern blot analyses. A value of $P<0.05$ was regarded as statistically significant for analyses using either the Student's t-test or 95% C.I..

RESULTS

The Gal-Lectin stimulates stable expression of TNF-α mRNA and TNF-α protein secretion

BMM stimulated with 100, 250 and 500 ng/ml of mAb affinity purified native Gal-lectin for 3 h increased the expression of TNF-α mRNA 3-, 6-, and 8-fold respectively, as compared to medium alone (Fig. 1). TNF-α mRNA levels increased 10-fold in BMM exposed to LPS (100 ng/ml) and only 2-fold in BMM exposed to pooled soluble amebic proteins (50 μg/ml) (Fig. 1, lane 3). Specificity for the Gal-lectin was shown by the addition of rabbit polyclonal anti-lectin serum which reduced TNF-α mRNA

expression in response to the Gal-lectin by 50% and in response to soluble amebic proteins by 40%, but had no effect on LPS-stimulated TNF-α mRNA levels (Fig. 2). Rabbit preimmune serum had no effect on any of the stimuli tested.

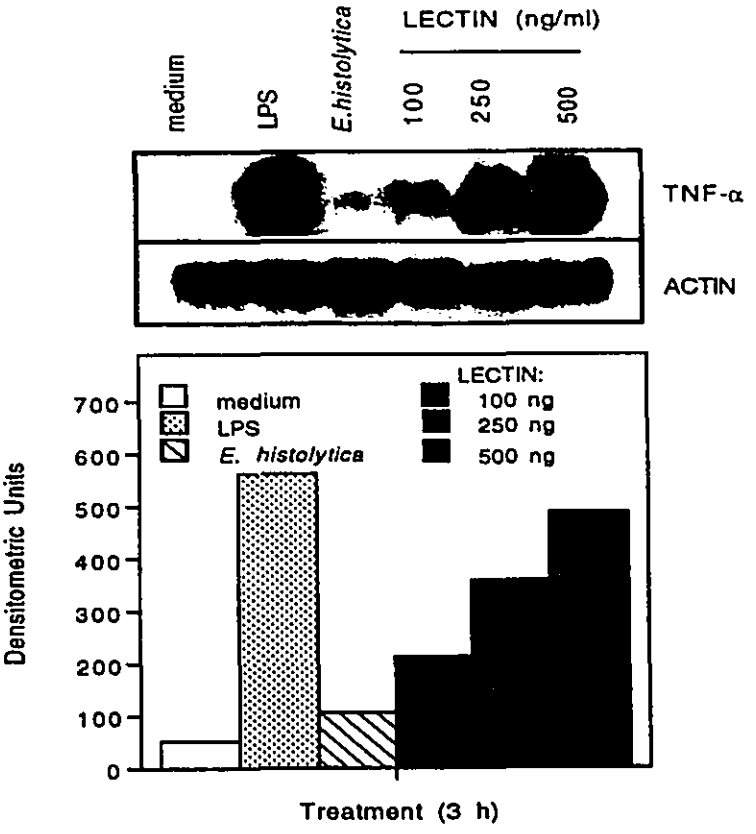

Figure 1. TNF-α mRNA expression in BMM exposed to LPS, soluble *E. histolytica* proteins and mAb affinity purified Gal-lectin. BMM were in the presence of medium alone or stimulated with LPS (100 ng/ml), soluble *E. histolytica* (50 μg/ml) proteins or purified amebic Gal-lectin for 3 h. Quantity of TNF-α mRNA was determined after normalization to actin levels and expressed as densitometric units in the histogram with identical lane designations for both Northern blot and histogram.

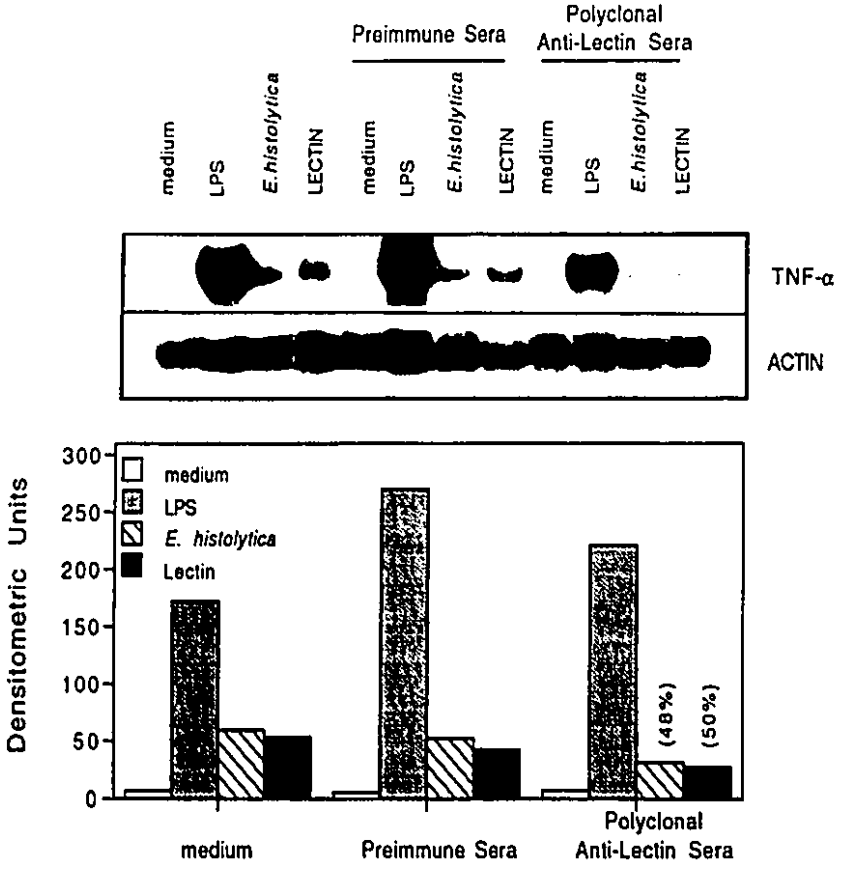

Figure 2. Polyclonal anti-lectin serum specifically inhibited Gal-lectin stimulated TNF-α mRNA expression in BMM. LPS (100 ng/ml), soluble *E.histolytica* proteins (50 μg/ml) or purified Gal-lectin (100 ng/ml) were treated with either medium, preimmune rabbit serum (1:1000 dilution) or polyclonal-anti lectin serum (1:1000 dilution) for 1 hr at 4°C prior to incubation with macrophages for 1 hr at 37°C. Results of scanning densitometric analysis of Northern blot are presented as a histogram with identical lane designations for both Northern blot and histograms. Values in parentheses show percentage inhibition relative to homologous controls.

We have previously shown that soluble amebic proteins (50 μg/ml) stimulate the unstable transcription of TNF-α mRNA and fail to induce TNF-α secretion in BMM (26). To determine if Gal-lectin stimulated TNF-α mRNA was similarly degraded, an actinomycin D (transcription inhibitor) stability experiment was performed using the stability of TNF-α mRNA in response to LPS as a standard. BMM stimulated for 3 h with LPS or Gal-lectin expressed increased levels of TNF-α mRNA that degraded slowly

up to 2 h (Fig. 3). The stability of the TNF-α mRNA (half-life of RNA remaining represented by 50% mark on linear slope, Fig. 3) in response to the Gal-lectin was similar to that of LPS-stimulated cells. Likewise, equivalent amounts of TNF-α (ELISA assay: 11, 26) were secreted by BMM exposed for 3 h to either 500 ng/ml Gal-lectin (41.73 ± 8.18 U/ml) or 100 ng/ml of LPS (48.83 ± 3.58 U/ml). BMM exposed to lower concentrations of the Gal-lectin (< 250 ng/ml), or to soluble amebic proteins did not secrete TNF-α.

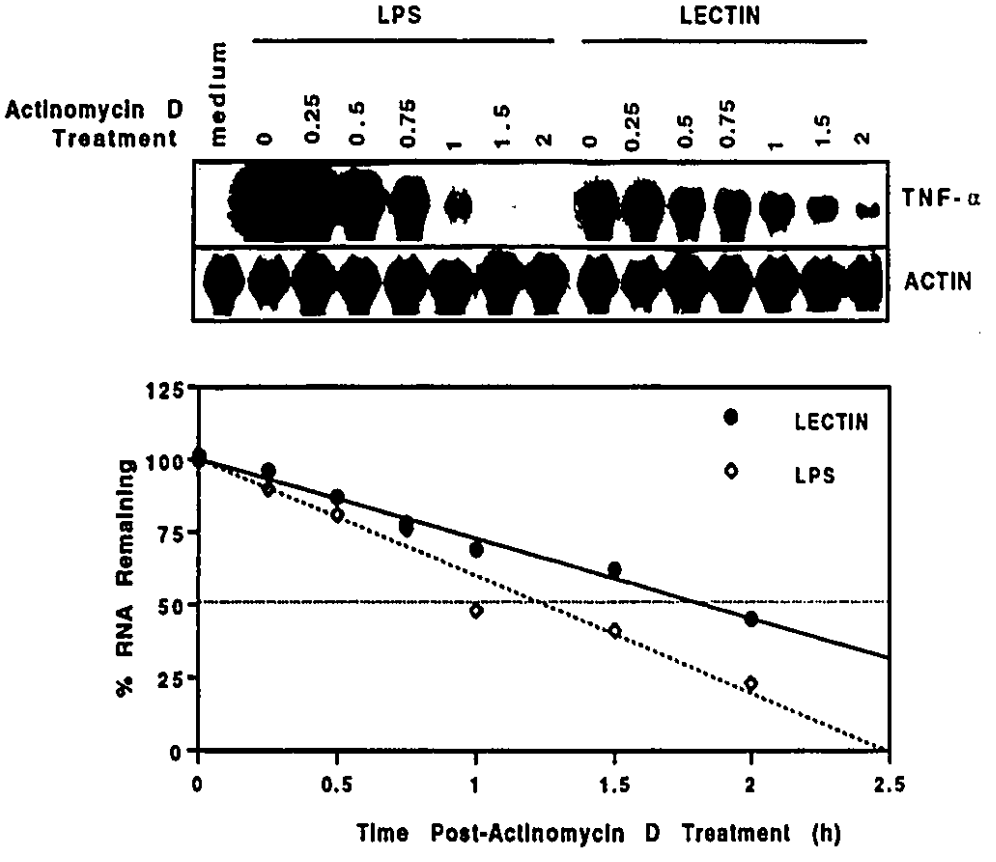

Figure 3. Stability of TNF-α mRNA in BMM stimulated with LPS or Gal-lectin. BMM were stimulated with either LPS (100 ng/ml) or Gal-lectin (100 ng/ml) for 3 h and then treated with actinomycin D (10 μg/ml, Gibco). Total RNA was isolated at various times following actinomycin D treatment. Densitometry of autoradiographs of Northern blots for both TNF-α and actin levels was performed. TNF-α mRNA levels are expressed as a percentage of the mRNA levels determined before the addition of actinomycin D (3 h post-stimulation). The $t_{½}$ (50% stability) was calculated from the linear graph for both LPS (r=0.980) and Gal-lectin (r=0.993)-stimulated BMM.

Anti-Gal-Lectin mAbs Inhibit Amebic Adherence to CHO Cells

As shown by Western blot ECL analysis (Fig. 4), rabbit polyclonal anti-lectin serum as well as anti-lectin mAbs 8C12, H85, and 1G7 recognized both the 150 kDa and the 170 kDa subunit of the Gal-lectin (27) whereas mAb 7F4 recognized only the 170 kDa subunit. Rabbit preimmune serum and anti-human colonic mucin mAb R35 did not react with either of the Gal-lectin isoforms.

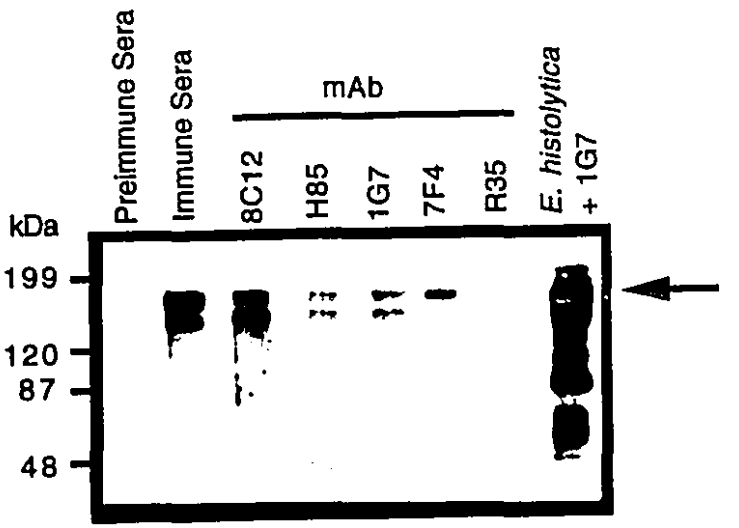

Figure 4. Polyclonal anti-lectin sera and mAbs recognize the 170 kDa heavy subunit of the lectin. Enhanced chemiluminescence (ECL) Western blot analysis was performed as described. The following antibodies were added to each lane: 1, rabbit preimmune sera; 2, rabbit anti-lectin immune sera; 3-6, mAbs against the Gal-lectin, 8C12, H85, 1G7 and 7F4; 7, irrelevant control mAb R35 which recognizes human colonic mucins (courtesy of Dr. D. Podolsky). The presence of the 170 kDa Gal-lectin subunit in soluble *E. histolytica* proteins (50 µg/ml, lane 8) recognized by mAb 1G7 is shown for comparison purposes. The arrow indicates the 170 kDa heavy subunit band resolved by ECL.

With a panel of anti-Gal-lectin mAbs we sought to identify the epitopes of the Gal-lectin that mediate adherence of trophozoites to CHO cells. Patients' immune sera were included in our study to demonstrate the presence of adherence-inhibitory antibodies following invasive amebiasis. Both human immune serum and rabbit polyclonal anti-lectin serum inhibited amebic adherence to CHO cells by 94% ($P<0.05$) as compared to

their homologous controls (Fig. 5). Similarly, anti-lectin mAbs 8C12, H85 and 1G7 inhibited amebic adherence by 24, 37 and 40% respectively ($P<0.05$), whereas mAb 7F4, which is also of the IGg2b isotype, did not (Fig. 5).

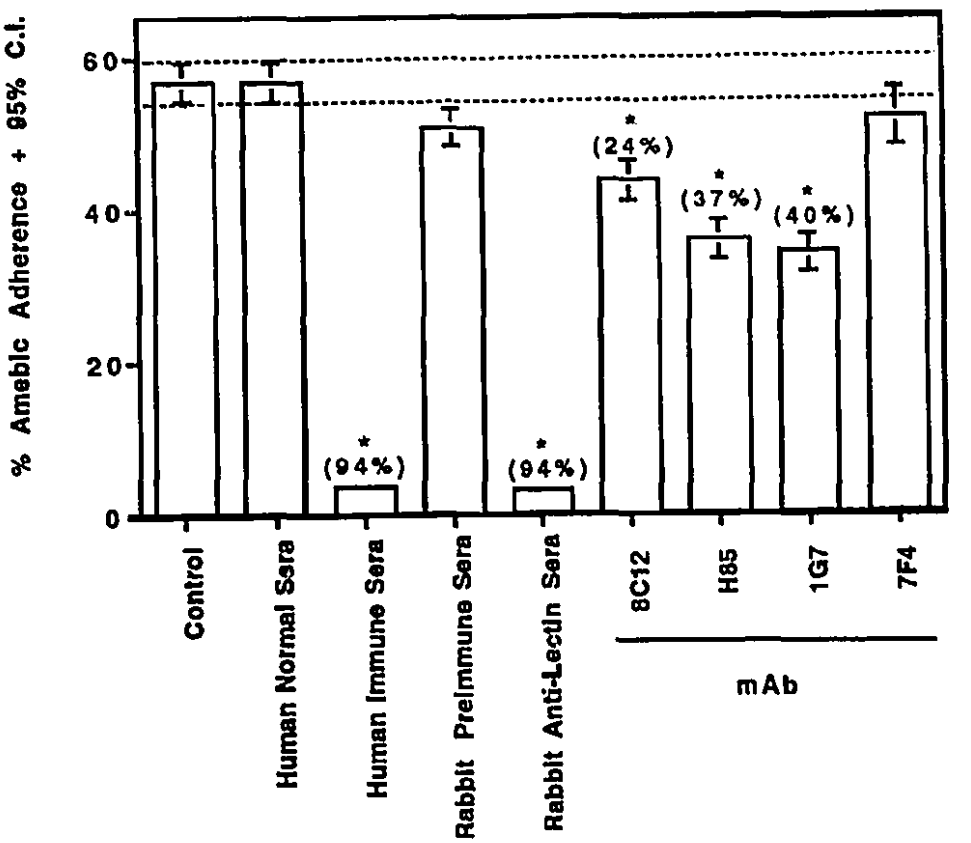

Figure 5. MAbs against the Gal-lectin inhibit amebic adherence to CHO cells. *E. histolytica* trophozoites (10^4) were incubated at 4°C for 1 hr with either medium, human serum (1/100 dilution of either uninfected or pooled immune serum from amebic liver abscess patients (courtesy of Dr. J. Keystone) or rabbit serum (1/100 dilution of preimmune and polyclonal anti-lectin) or mAbs 8C12, H85, 1G7, and 7F4 (10 μg/10^4 amebae) before being added to CHO cells (2 X 10^5) for 2 hr at 4°C. Statistical analysis used 95% confidence intervals, and (*) refers to significant difference from control levels at the $P<0.05$ level. Values in parentheses show percentage inhibition relative to homologous controls (n=18 for each).

Anti-Gal-Lectin mAbs Inhibit TNF-α mRNA Induction

To determine if the same adherence-mediating epitopes of the Gal-lectin were also involved in the induction of TNF-α mRNA, BMM were stimulated with LPS, soluble *E. histolytica* proteins or Gal-lectin in the presence of the anti-lectin mAbs and then

TNF-α mRNA expression was quantified by Northern blot analysis (Fig. 6). With the exception of mAb 7F4, the adherence-inhibitory anti-lectin mAbs 8C12, H85 and 1G7 inhibited TNF-α mRNA induction in response to the Gal-lectin by 28, 82, and 84%, respectively, as compared to BMM stimulated in the absence of mAb (medium) or to mAb 7F4. TNF-α mRNA expression in response to soluble *E. histolytica* protein was also similarly inhibited whereas the response to LPS was unaffected by any mAb.

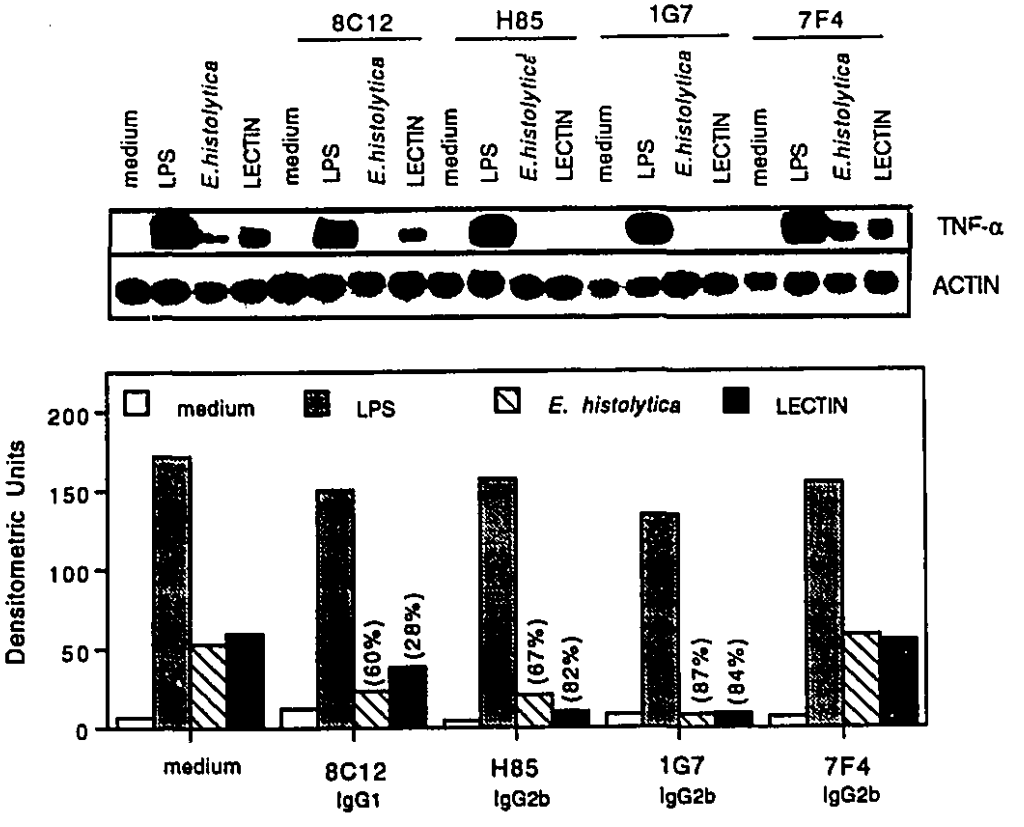

Figure 6. Anti-lectin mAbs inhibit TNF-α mRNA induction in BMM. BMM were stimulated under the same conditions as Figure 2 using 1 μg/ml affinity purified mAbs. Results of scanning densitometric analysis of Northern blots are presented as a histogram and are expressed as densitometric units with identical lane designations for both blots and histograms. Values in parentheses show percentage inhibition of the response relative to BMM in the presence of mAb 7F4 or medium alone.

DISCUSSION

Recent studies have shown that the purified native Gal-lectin (12) as well as a recombinant cysteine-rich portion of the 170 kDa lectin subunit (23) can confer 67-71% vaccine efficacy against amebic liver abscess formation in gerbils; however, the mechanism of protection has not been defined. The focus of this study was to identify the functional epitopes of the Gal-lectin 170 kDa subunit involved in amebic adherence and in the induction of TNF-α production for cell-mediated immunity against amebiasis. We have demonstrated that the Gal-lectin, in concentrations as low as 100 ng/ml, was sufficient to increase TNF-α mRNA levels in BMM by 3-fold. More importantly, TNF-α mRNA expression was stable and TNF-α protein was secreted in the culture medium similar to that observed in LPS stimulated cells. Therefore, unlike pooled soluble amebic proteins which down-regulate macrophage functions (26, 28-30), the native Gal-lectin activates naive macrophages.

The development of effective vaccines against any human parasitic disease has eluded researchers. The recent, albeit partial, success with the vaccine SPf66 against malaria (31) is not only the first vaccine against a parasitic disease of humans, but also shows the possibilities of using synthetic peptides successfully as a vaccine component. Limiting the vaccine to only peptides or portions of the molecule would reduce the risk of including epitopes which generate antibodies which may enhance the survival of the parasite. In the case of *E. histolytica* infections, patients produce antibodies which can either augment or inhibit amebic adherence to targets (21, 25). For example, two mAbs that recognize epitopes between aa 897-998 of the Gal-lectin 170 kDa subunit enhance amebic adherence to CHO cells and colonic mucins (20, 25). With the use of a panel of mAbs that map to the cysteine rich region of the 170 kDa subunit we have identified the portion of the Gal-lectin that mediates amebic adherence to CHO cells and stimulates the

induction of TNF-α mRNA by BMM *in vitro*. MAbs H85 and 1G7 inhibited amebic adherence to CHO cells by approximately 40% and inhibited TNF-α mRNA expression in macrophages exposed to the Gal-lectin by >80%. MAb H85 recognizes a site within aa 1033-1082 and mAb 1G7 recognizes a site within aa 596-818. Interestingly, mAb 8C12 whose recognition site (aa 895-998) lies between those of H85 and 1G7, only modestly inhibited amebic adherence (24%) and slightly reduced TNF-α mRNA expression (28%). The three dimensional conformation of the Gal-lectin could account for the effect observed by aa 596-818 and then the span to aa 1033-1082. As mAb 7F4 had no effect on inhibiting either amebic adherence or TNF-α mRNA induction in response to the Gal-lectin, the region containing its recognition site (aa 1082-1138) may be beyond the area which mediates any biological effects of the molecule. From these results we have defined a region located between amino acids 596-1082 of the 170 kDa subunit of the Gal-lectin which is involved in both amebic adherence and stimulation of TNF-α mRNA expression by BMM. Recently, a recombinant fusion protein of the Gal-lectin containing amino acids 758-1134 yielded 71% efficacy as a vaccine to prevent the formation of amebic liver abscesses in gerbils (23).

E. histolytica evade humoral responses by capping and shedding IgG (32) or by proteolytic cleavage of sIgA (33). Thus, it is not unusual that intestinal colonization with *E. histolytica* occurs in immune individuals (2), in spite of high titres of anti-Gal-lectin IgG (21) and sIgA antibodies (34, 35). *In vitro*, the Gal-lectin has been shown to activate T-cells for IFN-γ production, a cytokine which primes macrophages (22). It can be hypothesized that if colonic ulceration occurs in drug-cured patients or following Gal-lectin immunization, the sensitized T lymphocyte population in the lamina propria will produce IFN-γ to prime macrophages for enhanced TNF-α and NO production which are necessary for host defence against amebic invasion. The Gal-lectin can stimulate NO

production from IFN-γ-primed-macrophages for cytotoxicity against *E. histolytica* (unpublished observations).

In summary, we have shown that the Gal-lectin can stimulate a cell-mediated immune response *in vitro*, as measured by the increased levels and stability of TNF-α mRNA and secretion of TNF-α protein into culture medium. Furthermore, through the use of a panel of Gal-lectin mAbs we have identified a region of the molecule (aa 596-1082) which mediates *E. histolytica* adherence to CHO cells and stimulates TNF-α mRNA expression in BMM. This region, containing the amebic binding domain and proinflammatory sites, holds promise for use as a subunit vaccine candidate.

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CONNECTING STATEMENT II

In manuscript II it was shown that a single purified amebic glycoprotein, the native Gal-lectin, was capable of stimulating stable TNF-α mRNA expression and protein production by naive macrophages. Furthermore, with the use of the panel of monoclonal antibodies, a region of 487 aa of the 170 kDa heavy subunit of the Gal-lectin was identified which mediated amebic adherence to a target cell and was responsible for inducing TNF-α mRNA expression by macrophages. Despite the ability of the Gal-lectin to stimulate TNF-α secretion by macrophages, the Gal-lectin failed to activate naive macrophages for either NO production or amebicidal activity. Macrophages are activated by a cascade of stimuli given in a defined sequence. Macrophages primed with IFN-γ yield enhanced levels of NO in response to bacterial components such as LPS. We next determined, therefore, if the Gal-lectin could stimulate IFN-γ primed macrophages for NO production and amebicidal activity. IFN-γ was chosen as the priming signal because T-lymphocytes isolated from either patients or animals cured of amebic liver abscesses have been shown to produce IFN-γ in response to Gal-lectin *in vitro*.

MANUSCRIPT III

The Galactose-Adherence Lectin of *Entamoeba histolytica* Activates IFN-γ Primed Macrophages for Amebicidal Activity Mediated by Nitric Oxide*[1]

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*Submitted for publication to the *Journal of Immunology*

ABSTRACT

Entamoeba histolytica adhere via the galactose-inhibitable lectin (Gal-lectin) to human colonic mucins and intestinal epithelial cells *in vitro* as a prerequisite to amebic invasion. The Gal-lectin is a protective antigen in the gerbil model of amebiasis and *in vitro* stimulates interferon-gamma (IFN-γ) production by sensitized T-lymphocytes and tumor necrosis factor-alpha (TNF-α) production by naive bone marrow macrophages (BMM) *in vitro*. Resistance to amebiasis appears to require an effective cell-mediated immune response against *E. histolytica* trophozoites mediated primarily by nitric oxide (NO) released from activated macrophages. In this study we demonstrate that the Gal-lectin (100-500 ng/ml) stimulated TNF-α and inducible nitric oxide synthase (iNOS) mRNA expression in IFN-γ primed BMM similar to that of LPS (100 ng/ml)-exposed cells. Primed BMM produced TNF-α and NO in response to the Gal-lectin in a dose-dependent manner. Anti-lectin monoclonal antibody 1G7 which recognizes a domain (amino acids 596-818) within the TNF-α mRNA stimulating region of the Gal-lectin, specifically inhibited TNF-α and iNOS mRNA induction and TNF-α and NO production by primed BMM in response to Gal-lectin (100 ng/ml). Simultaneous treatment of BMM with IFN-γ and Gal-lectin (100 ng/ml) activated the cells to kill *E. histolytica* trophozoites whereas IFN-γ treatment alone had no effect. In the presence of mAb 1G7 and aminoguanidine (a selective iNOS inhibitor) NO production and amebicidal activity were inhibited >80%. These results suggest that the TNF-α stimulating region of the native Gal-lectin is a potent stimulus for NO production in IFN-γ primed BMM which may be essential for host defense against amebiasis.

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INTRODUCTION

Present evidence in both human and animal models of *Entamoeba histolytica* infections supports the idea that limitation and prevention of recurrent invasive amebiasis requires the development of an effective cell-mediated immune response (1-11). Activated macrophages kill *E. histolytica* trophozoites *in vitro* in a contact-dependent manner (3), primarily through the production of nitric oxide (NO), with minor contributions by cytolytic proteases and reactive oxygen intermediates (3, 5, 6). TNF-α, produced by activated macrophages, acts in an autocrine fashion to enhance inducible nitric oxide synthase (iNOS) gene expression and subsequently, NO production (6, 12). Furthermore, anti-TNF-α antibody inhibits TNF-α release, NO production and amebicidal activity by activated murine macrophages (6).

E. histolytica adhere to target cells and may be a necessary prerequisite for cytolytic activity (13). Amebic adherence to human leukocytes, mammalian tissue culture cells, rat and human colonic mucosa or purified colonic mucins (7, 13-15) is mediated by the cysteine rich region of the 170 kDa heavy subunit of the galactose-inhibitable lectin (Gal-lectin) (15, 16) . The Gal-lectin is a 260 kDa heterodimeric glycoprotein comprised of a 170 kDa heavy subunit and a 35 kDa light subunit linked together by disulphide bonds (16). The 170 kDa heavy subunit is an immunodominant antigen, recognized by the immune sera of >90% of patients worldwide with amebic liver abscess (17). Native Gal-lectin demonstrated a 67% efficacy as a protective antigen when used in the gerbil model of amebic liver abscess (18) and two independent studies demonstrated that recombinant fusion proteins which included the highly immunogenic, cysteine-rich extracellular portion of the 170 kDa subunit similarly protected gerbils upon challenge with 71% (19) and 81% efficacy (20). The immunological basis for the protective immune response elicited by the Gal-lectin is unknown; however the Gal-lectin is capable

of stimulating cell-mediated immune responses *in vitro*. Lymphocytes isolated from either Gal-lectin immunized animals or from patients drug-cured of amebiasis, proliferated (8, 21-23) and produced IL-2 and IFN-γ (21, 22) in response to the Gal-lectin. We recently reported that a region between amino acids 596-1082 of the cysteine rich region of the 170 kDa Gal-lectin was responsible for stimulating murine bone marrow-derived macrophages (BMM) for stable TNF-α gene expression and protein production (24). As TNF-α enhances NO production (6, 12), which is cytotoxic against *E. histolytica* (5, 6), we hypothesize that the TNF-α stimulating region of the Gal-lectin may be able to activate BMM. It is crucial to identify the regions of the Gal-lectin that activate macrophages as it is the most promising subunit vaccine candidate against amebiasis.

In this study we determined whether the TNF-α stimulating region of native Gal-lectin can activate IFN-γ primed BMM for NO production and amebicidal activity. Herein, we demonstrate that the Gal-lectin can activate IFN-γ-primed BMM for enhanced TNF-α and iNOS mRNA expression with a corresponding increase in TNF-α and NO production. Furthermore, simultaneous treatment of BMM with IFN-γ and Gal-lectin activated macrophages for NO-dependent amebicidal activity.

MATERIALS AND METHODS

Reagents. RPMI 1640 medium with L-glutamine was purchased from Gibco/BRL Life Technologies (Burlington, Ontario, Canada) and was supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah; complete medium). Recombinant TNF-α, rabbit anti-mouse TNF-α polyclonal antibody and hamster anti-mouse TNF-α monoclonal antibody used for TNF-α ELISA were purchased from Genzyme (Cambridge, Mass.). LPS (*Escherichia*

coli serotype 0111:B4, phenol extract), sulfanilamide, N-1-naphthylethylenediamine dihydrochloride and aminoguanidine hemisulfate salt were obtained from Sigma (St. Louis, Mo.).

Cultivation of E. histolytica. E. histolytica (strain HM1-IMSS) originally provided by L. Diamond (National Institutes of Health, Bethesda, Md.) was cultured in our laboratory as previously described (3). *E. histolytica* trophozoites in mid-log phase (3 days) were used in all studies.

Preparation of BMM. BMM were isolated from the femurs and tibia of 42 day old female BALB/c mice (Charles River, Canada). Femurs and tibia were flushed using a 27 gauge needle and syringe with complete RPMI medium. Cells were cultured overnight on adherent petri dishes (Becton Dickinson, Lincoln Park, N.J.) in complete RPMI medium with 20% (vol/vol) L929-conditioned medium as a source of colony stimulating factor-1. The following day, non-adherent cells were transferred to non-adherent plates (Baxter Diagnostics Corporation, Mississauga, Ontario, Canada) and cultured for 6 days in the presence of 15% (vol/vol) L929-conditioned medium in complete RPMI medium. After 6 days, the cells were washed with Dulbecco's phosphate-buffered saline (pH 7.2; Gibco) and quiesced (1 X 10^6 cells/ml) for 12-18 h in CSF-1-free complete RPMI medium.

Preparation of soluble amebic proteins and Gal-lectin. Soluble amebic proteins were prepared following freeze-thaw lysis of trophozoites. The lysed cells were centrifuged (15, 000 X *g*, 5 min, 4°C) to remove cellular debris. The supernatant containing the soluble amebic proteins was collected and the protein concentration was quantified by the method of Bradford (25) using bovine serum albumin as a standard. The isolation and purification of native Gal-lectin by mAb affinity chromatography has been described in detail, elsewhere (16). Briefly, octylglucoside (Sigma) solubilized

amebae were applied to a column consisting of immobilized purified anti-lectin mAbs H85, 7F4, 5B8, 3F4, and 6D2 (16). The purified Gal-lectin (1 µg/µl) and freshly prepared soluble *E. histolytica* proteins (50 µg/ml) contained undetectable endotoxin contamination as measured by the ETOXATE-assay (sensitive to 0.05-0.5 endotoxin units per ml, Sigma).

RNA isolation and Northern blot analysis. Total RNA from BMM was isolated with Trizol, (Gibco) a variation of the guanidinium thiocyanate-phenol-chloroform method (26). RNA samples (10 µg) were denatured for 1 h at 50°C in the presence of glyoxal prior to electrophoresis in a 1% agarose gel and then transferred to a nylon membrane. Northern blotting was performed by incubating ^{32}P-labelled cDNA probes with the membranes for 12-18 h at 42°C as described (24, 27). The membranes were washed and then exposed to Kodak XAR-5 film with an intensifying screen for 12-18 h at -70°C. Optical density of the bands was analysed by scanning densitometry (ISCO UA-5 absorbance/fluorescense detector, ISCO Inc., Lincoln, Nebraska) to determine the concentration of probe bound to the membrane. To ensure equal loading, all blots were stripped of bound probe and rehybridized with an actin probe as an internal control. The TNF-α probe was the 1.5 kb *Pst*I fragment from pmTNF-1 from W. Fiers, University of Ghent, Belgium and the murine iNOS probe was the 4.1 kb *Not*I fragment from pmmac-NOS supplied by C.J. Lowenstein, John Hopkins University School of Medicine, Baltimore, MD. The actin probe consisted of the 1.25 kb *Pst*I fragment of pBA-1.

TNF-α ELISA. TNF-α production was measured by an ELISA method described elsewhere (6, 27, 28). Briefly, ELISA plates were coated with hamster anti-mouse TNF-α mAb at 4°C overnight. The plates were washed with PBS-Tween (0.05% Tween 20 in 0.01 M PBS) and incubated for 1 h at 37°C with 2% bovine serum albumin in 0.01 M PBS. After washing, recombinant mouse TNF-α (2 to 256 U/ml for standard curve) or

macrophage culture supernatants were placed into the wells and incubated for 1 h at 37°C. The plates were washed and rabbit anti-mouse TNF-α polyclonal antibody was added. The plates were incubated for 1 h at 37°C, washed and incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate for 1 h at 37°C. Following washes, plates were incubated with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (ABTS) (Bio-Rad Laboratories, Mississauga, Ont.) for 20 min and then the optical density was measured on an ELISA reader at 415 nm. Data are expressed as U/ml/10^6 cells.

Measurement of Nitric Oxide production. Culture supernatants (50 µl) were assayed for NO_2^- by the Griess reaction (29). Briefly, an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) was incubated with macrophage supernatnants for 10 min at room temperature and the absorbance was measured at 550 nm in an ELISA reader (Biotecx Instruments, Mandel Scientific, Ontario, Canada). NO_2^- concentration was determined by using sodium nitrite as a standard. Data are expressed as total micromolar NO_2^- produced by 10^6 cells following 24 hr stimulation with various treatments as indicated.

Assessment of amebicidal activity by activated macrophages. BMM were stimulated simultaneously with IFN-γ (100 U/ml) and either LPS (100 ng/ml), *E. histolytica* soluble proteins (50 µg/ml) or Gal-lectin (100 ng/ml) for 24 h. In some cases the stimulating agents were initially incubated for 1 h on ice with either mAb 1G7 or R35 (1 µg/ml) before being added to BMM. Aminoguandine (5 mM), an inhibitor of NO, was added to some cell cultures at the time of stimulation. BMM (1 X 10^6/ml) and *E. histolytica* trophozoites (1 X 10^4/ml) were suspended in complete RPMI 1640 medium in plastic tubes (12 X 75 mm). Cells were centrifuged at 150 *g* for 10 min and then incubated at 37°C in 5%CO_2 for 6 h. After incubation, the tubes were placed on ice and the percent viability of the trophozoites was assessed by trypan blue exclusion (0.1%

trypan blue in PBS) by counting > 100 amebae/tube. The data were compared to the viability of amebae incubated alone for 6 h then adjusted to the percent of amebic killing by macrophages.

Statistics. All Northern blots were performed three times with a single representative experiment shown. The results of TNF-α ELISA and NO/Griess reaction were analysed by the Student's *t*-test with a probability value of $P<0.05$ regarded as statistically significant (n=9, 3 experiments with 3 replicates). Amebicidal activity of macrophages were calculated with 95% confidence intervals (n=6).

RESULTS

The Gal-lectin stimulates the expression of iNOS and TNF-α mRNA in IFN-γ-primed BMM

NO is the major effector molecule produced by activated macrophages for killing *E. histolytica* trophozoites *in vitro* (5, 6). In murine macrophages, NO production is tightly regulated by the gene expression, synthesis and activity of iNOS (30) and by TNF-α (6, 12). As the Gal-lectin can stimulate TNF-α mRNA and TNF-α production in naive BMM (24) we investigated the effect of the Gal-lectin on iNOS and TNF-α mRNA expression in IFN-γ primed BMM. BMM were primed with IFN-γ (100 U/ml for 24 h), followed by 3 or 24 h stimulation with either LPS (a positive control), soluble *E. histolytica* proteins (50 μg/ml) or increasing concentrations of Gal-lectin (100, 250 and 500 ng/ml) and the levels of iNOS and TNF-α mRNA determined by Northern blotting. Stimulation of primed BMM with LPS or Gal-lectin (100-500 ng/ml) doubled the levels of iNOS mRNA expressed as compared to the level expressed by BMM in response to IFN-γ alone (Fig. 1A, open bars). The levels of iNOS mRNA were enhanced equally by either LPS (100 ng/ml) or Gal-lectin (500 ng/ml). In response to soluble *E. histolytica*

proteins, there was a marginal increase in the levels of iNOS mRNA. To demonstrate specificity of the Gal-lectin, all stimuli were preincubated for 1 h at 4°C with 1 μg/ml of either mAb 1G7 (Fig. 1a, solid bars) which recognizes aa 596-818 of the 170 kDa heavy subunit of the Gal-lectin and inhibits TNF-α mRNA induction (24), or mAb R35 (Fig. 1a, shaded bars) which recognizes an irrelevant glycoprotein (human colonic mucins) as a control. As TNF-α can upregulate iNOS mRNA and NO production (6, 12), BMM were treated with the selective mechanism-based inhibitor of iNOS (31), aminoguanidine (AG, 5 mM). As shown in Fig. 1A (solid bars), mAb 1G7 specifically inhibited the induction of iNOS mRNA expression by 60 and 32% in response to 100 and 250 ng/ml of Gal-lectin respectively (Fig. 1A), while mAb R35 and AG had no suppressive effects. Neither mAb 1G7, nor AG treatment altered the level of iNOS mRNA in response to 500 ng/ml of Gal-lectin whereas mAb R35 slightly increased the expression of iNOS (Fig. 1a). Interestingly, mAb 1G7 and R35 augmented the iNOS mRNA in IFN-γ primed BMM stimulated with LPS. As AG did not alter iNOS mRNA expression its inhibitory effects are not exerted at the level of iNOS transcription.

After 24 h stimulation, IFN-γ-primed BMM continued to express iNOS mRNA in response to LPS or Gal-lectin stimulation (Fig. 1B, open bars), albeit at reduced levels as compared to the levels observed following 3 h stimulation. However, Gal-lectin (500 ng/ml) stimulation of iNOS mRNA was no longer equal to the level observed in response to LPS. mAb 1G7 markedly inhibited (72%) the induction of iNOS mRNA in response to 100 ng/ml of Gal-lectin and only slightly reduced (10-20%) in response to higher concentrations of Gal-lectin. The effects of mAb R35 and AG were variable and augmented the expression of iNOS in the presence of high concentrations of Gal-lectin (250-500 ng/ml). Therefore, even after 24 h the induction of iNOS mRNA by Gal-lectin stimulation (100 ng/ml) was specifically inhibited by the presence of anti-lectin mAb 1G7.

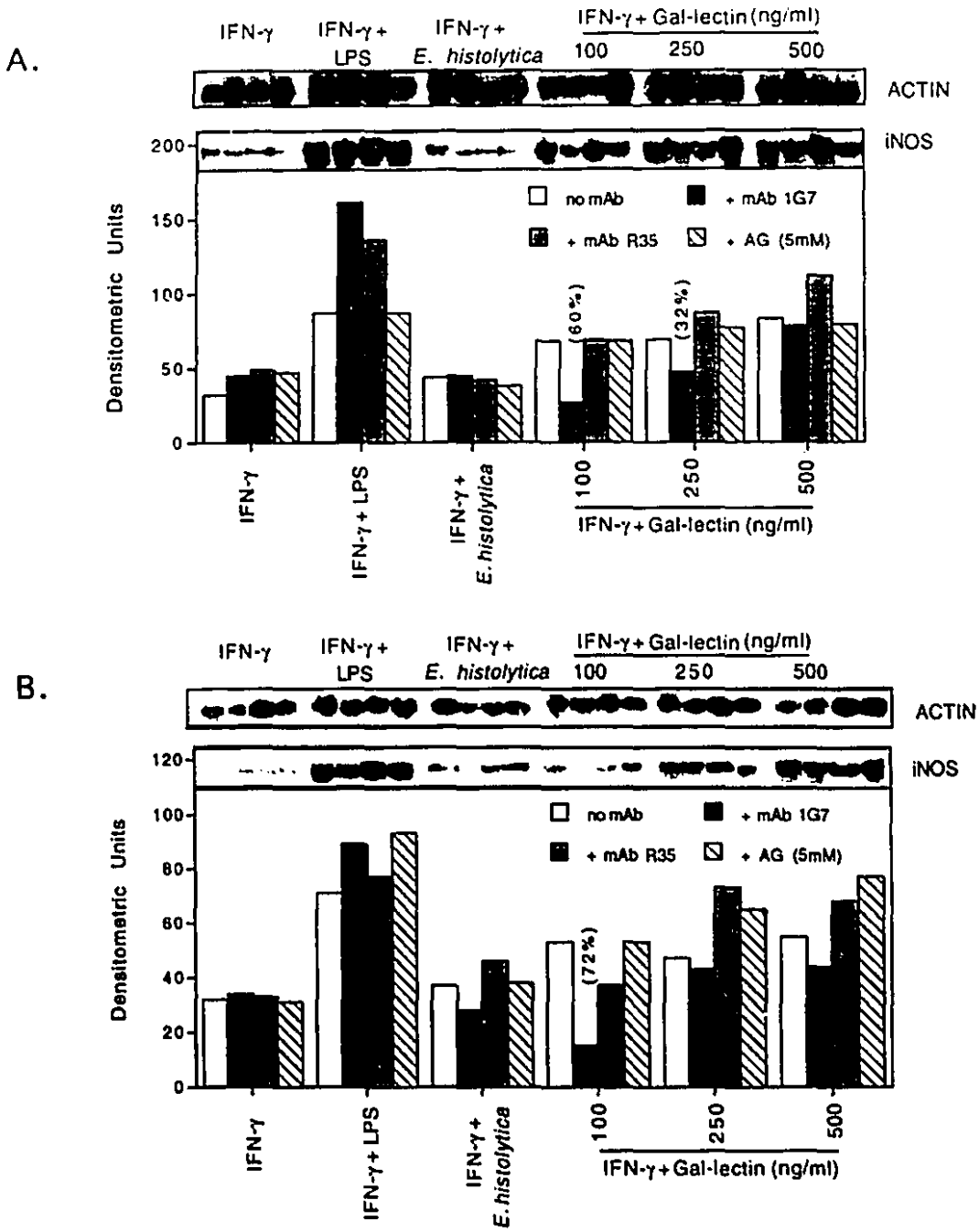

Figure 1. Gal-lectin stimulated iNOS mRNA expression in IFN-γ primed BMM is specifically inhibited by anti-lectin mAb 1G7. BMM were in the presence of medium alone or stimulated with LPS (100 ng/ml), soluble *E. histolytica* proteins (50 μg/ml) or Gal-lectin (100-500 ng/ml) for 3 h (A) or 24 h (B). The stimulating agents were incubated for 1 h at 4°C in the presence of either anti-lectin mAb 1G7 or control mAb R35 (1 μg/μl). AG (5mM) was added simultaneously with the stimulating agents to the primed BMM. Quantity of iNOS mRNA was determined after normalization to actin levels and expressed as densitometric units in the histogram.

As TNF-α enhances iNOS gene expression (6, 12), we also measured the levels of TNF-α mRNA produced in primed BMM after 3 h under the same experimental conditions as described above. IFN-γ treatment alone was insufficient to activate macrophages for enhanced TNF-α mRNA expression (Fig. 2). However, expression of TNF-α mRNA was nearly doubled in IFN-γ primed BMM exposed to LPS or Gal-lectin (100-500 ng/ml) (Fig. 2, open bars). The addition of soluble *E. histolytica* proteins increased the levels of TNF-α mRNA by 20%. Similar to iNOS mRNA, there was increased expression of TNF-α mRNA in BMM stimulated with LPS in the presence of mAb 1G7, mAb R35 and AG. However, mAb 1G7 inhibited *E. histolytica* and Gal-lectin (100 ng/ml) induced TNF-α mRNA expression by 39 and 37% respectively.

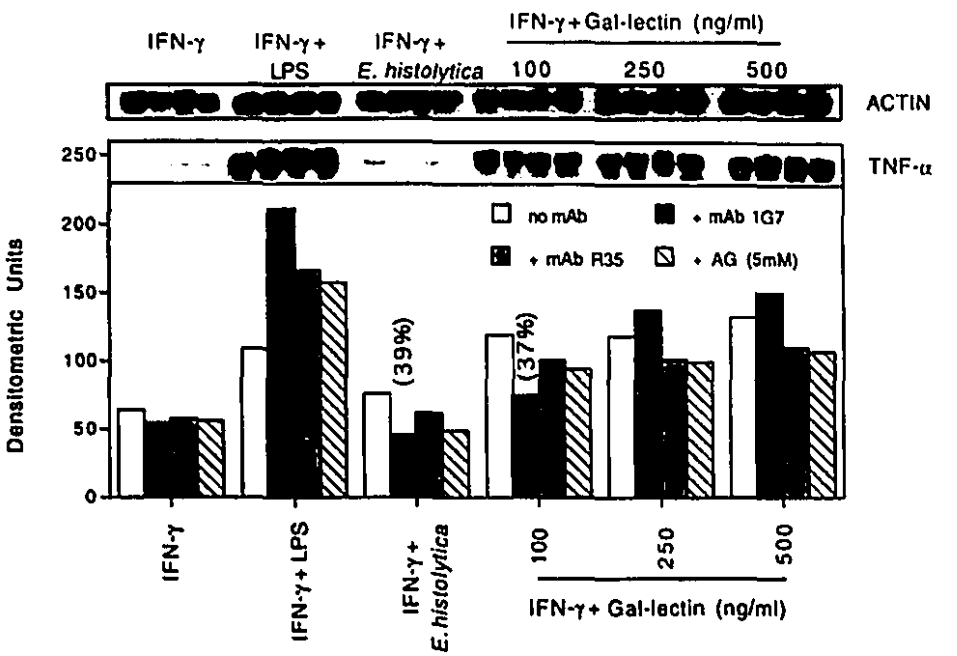

Figure 2. Gal-lectin stimulated TNF-α mRNA expression in IFN-γ primed BMM is specifically inhibited by anti-lectin mAb 1G7. BMM were in the presence of medium alone or stimulated with LPS (100 ng/ml), soluble *E. histolytica* proteins (50 μg/ml) or Gal-lectin (100-500 ng/ml) for 3 h. The stimulating agents were incubated for 1 h at 4°C in the presence of either anti-lectin mAb 1G7 or control mAb R35 (1 μg/μl). AG (5mM) was added simultaneously with the stimulating agents to the primed BMM. Quantity of TNF-α mRNA was determined after normalization to actin levels and expressed as densitometric units in the histogram.

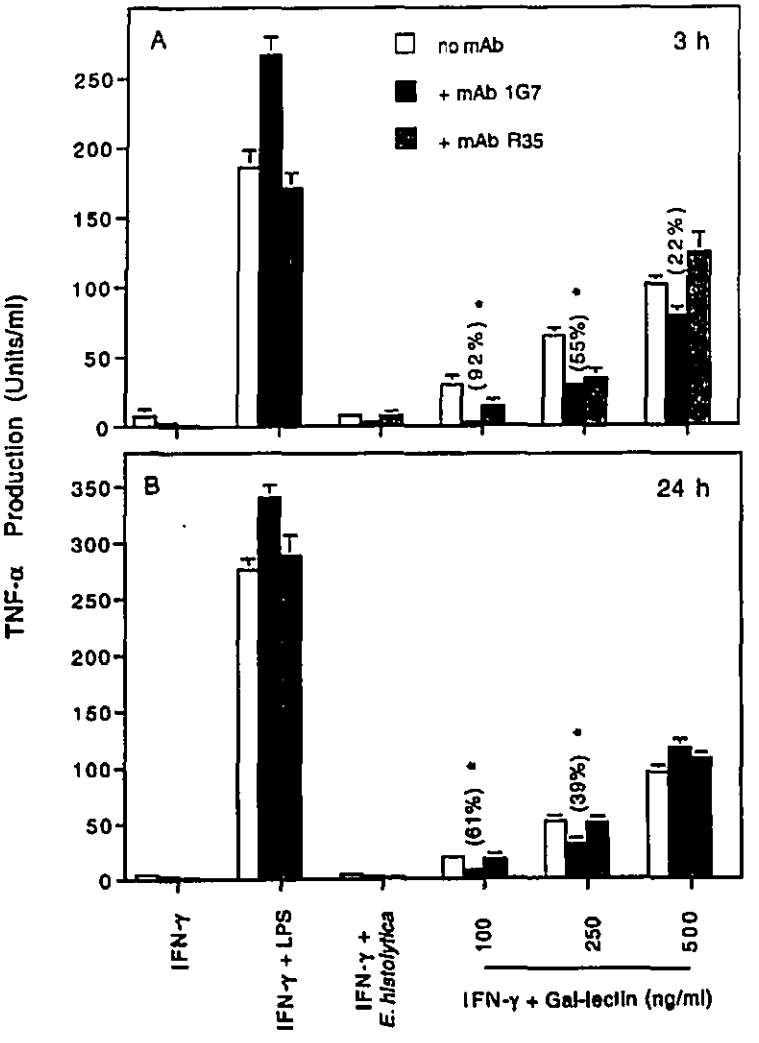

Figure 3. TNF-α production by Gal-lectin stimulated primed BMM is specifically inhibited by mAb 1G7. The levels of TNF-α secreted into the culture supernatant as described in Figure 2 for 3h (A) or 24h (B) were assayed by ELISA. Data are means + S.E. (n=9) * $P<0.05$ as compared to homologous controls (no mAb). With 100 ng/ml Gal-lectin mAb 1G7 significantly inhibited ($P<0.05$) TNF-α production at 3 and 24h compared to mAb R35.

The Gal-lectin activates IFN-γ-primed BMM for TNF-α and NO production

As there is a strong correlation between TNF-α production and the release of NO by activated macrophages (6, 12) we measured TNF-α secretion by primed BMM following 3 and 24 h exposure as described above. BMM stimulated with either IFN-γ alone or with *E. histolytica* proteins failed to produce TNF-α (Fig. 3A). In contrast,

primed BMM which were stimulated with LPS (3 h) demonstrated a 23-fold increase in TNF-α secretion. The Gal-lectin stimulated primed BMM in a dose-dependent manner for TNF-α secretion with 3-, 7- and 12-fold increase above control levels (Fig. 3A, open bars). TNF-α secretion in response to LPS stimulation was enhanced by 44% in the presence of mAb 1G7 and unaffected by mAb R35. The presence of mAb 1G7 but not mAb R35 significantly inhibited TNF-α secretion by 92 and 55% in response to a 100 and 250 ng/ml of Gal-lectin, respectively. As a 3 h stimulation period may not be sufficient for maximal TNF-α secretion, primed BMM were stimulated for 24 h as described above and TNF-α levels in the supernatant were quantified. Under these conditions neither IFN-γ alone or soluble *E. histolytica* proteins stimulated primed BMM for TNF-α production, whereas a 58-fold increase in TNF-α levels occurred in response to LPS (Fig. 3B). Similar to the observations following 3 h treatment, Gal-lectin increased TNF-α secretion in a dose-dependent manner by 3-, 10- and 19-fold (Fig. 3B). Again, TNF-α secretion in response to LPS was slightly increased in the presence of mAb 1G7, but not by mAb R35. Even after 24 h, mAb 1G7 but not mAb R35 significantly ($P<0.05$) inhibited TNF-α production in response to 100 and 250 ng/ml of Gal-lectin by 61 and 39% respectively.

The levels of NO_2^-, an end product of the NO reaction, were quantified as an indirect measurement of NO production. Following 3 h exposure of any of the stimuli tested primed BMM did not produce NO (data not shown). However, when BMM were exposed for 24 h to LPS, a 48-fold increase in NO_2^- levels was observed as compared to the levels with IFN-γ alone (Fig. 4). Stimulation of primed BMM with Gal-lectin (100-500 ng/ml) increased NO_2^- release in a dose dependent manner by 14-, 28-, and 35-fold (Fig. 4, open bars). The presence of AG, which did not affect iNOS mRNA (Fig. 1A/B), abrogated NO_2^- production by primed BMM in response to both LPS and Gal-lectin (Fig. 4, hatched bars). LPS-induced NO_2^- production was not affected by mAbs 1G7 and

R35. However, mAb 1G7 significantly inhibited NO_2^- production in response to 100, 250 and 500 ng/ml Gal-lectin by 52, 28 and 27% respectively. These data clearly demonstrate that the Gal-lectin can stimulate NO production by primed BMM which is partially inhibited specifically by mAb 1G7.

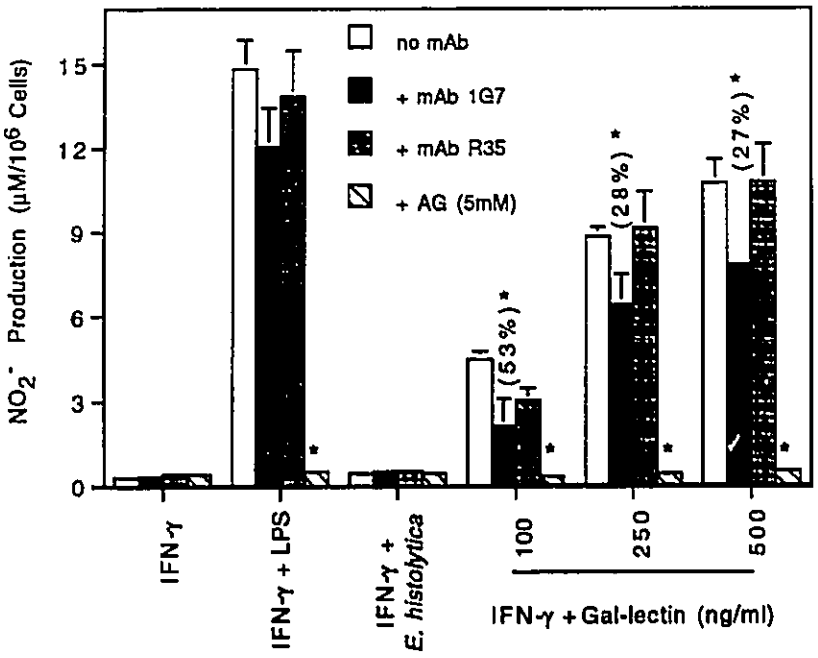

Figure 4. Gal-lectin stimulated NO_2^- production by primed BMM is specifically inhibited by mAb 1G7 and AG. Culture supernatants of primed BMM treated as described in Fig. 1B were assayed for NO_2^- production by the Griess reaction. Data are means + S.E. (n=9). * $P<0.05$ as compared to homologous controls (no mAb), $P>0.05$ for mAb 1G7 compared to mAb R35.

The Gal-lectin activates IFN-γ-primed macrophages for amebicidal activity

As the Gal-lectin can activate IFN-γ primed BMM for TNF-α and NO production we were interested in determining whether the Gal-lectin in the presence of IFN-γ (24 h) can activate naive BMM for cytotoxicity against *E. histolytica*. As shown in Fig. 5, BMM treated with IFN-γ alone or IFN-γ + *E. histolytica* proteins were not activated to kill amebae *in vitro* (Fig. 5, open bars). However, treatment with IFN-γ + LPS or IFN-γ + Gal-lectin activated BMM to kill 81 and 53% of *E. histolytica* trophozoites respectively.

Amebicidal activity of BMM stimulated with IFN-γ + LPS was unchanged in the presence of either mAb 1G7 (Fig. 5, solid bars) or mAb R35 (Fig. 5, shaded bars) whereas treatment with the iNOS inhibitor, AG, significantly reduced amebicidal activity by 47% (Fig. 5, hatched bars). Similarly, an 88 and 83% reduction in the killing activity of BMM stimulated with IFN-γ + Gal-lectin was observed in the presence of mAb 1G7 or AG treatment respectively, while the presence of mAb R35 had no effect. Of note, both mAbs did not inhibit but slightly increased the killing of amebae in IFN-γ + *E. histolytica* proteins-stimulated BMM which was inhibited in the presence of AG.

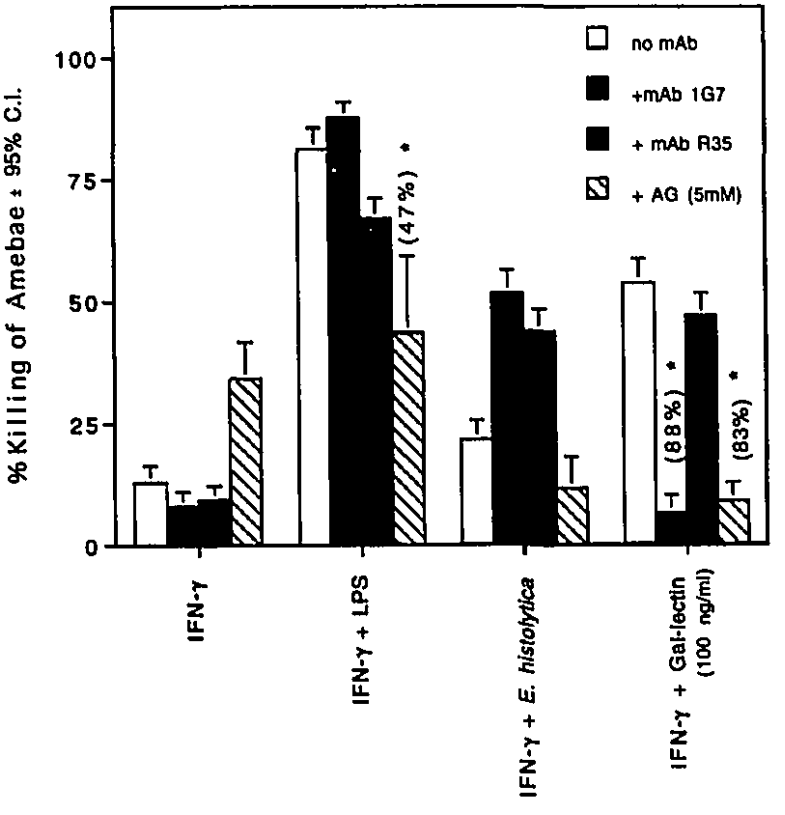

Figure 5. The Gal-lectin + IFN-γ activates BMM to kill *E. histolytica* trophozoites. BMM were treated with IFN-γ (100 U/ml) and either LPS (100 ng/ml), soluble *E. histolytica* proteins (50 µg/ml) or Gal-lectin (100 ng/ml) at 37°C for 24 h prior to incubation with trophozoites (target:effector ratio, 1:100) at 37°C for 6 h. All stimulating agents were preincubated with mAb 1G7 or R35 (1 µg/ml) as described in *Materials and Methods* before added to BMM. AG treatment of BMM occurred simultaneously with the addition of the stimulating agents. Amebic viability was measured by trypan blue exclusion (0.1% of trypan blue in PBS). Data are means + 95% C.I. (n=6). * $P<0.05$ as compared to homologous controls and mAb R35.

These results demonstrate that the reduction of amebicidal activity mediated by IFN-γ + Gal-lectin is dependent on the stimulation of NO similar to that of IFN-γ + LPS-activated BMM.

DISCUSSION

Acquired resistance to invasive amebiasis appears to require the development of an effective cell-mediated response (1-11), mediated primarily by activated macrophages. We have previously demonstrated that the *in vitro* killing of *E. histolytica* trophozoites by activated macrophages is accompanied by increased levels of NO (5). Furthermore, inhibition of NO by the addition of a competitive NO synthase inhibitor, L-NMMA, abrogated amebicidal activity of the macrophages (5). These data emphasize the importance of NO as the cytotoxic molecule produced by activated macrophages to kill *E. histolytica*. We (6) and others (12) have shown that TNF-α produced by activated macrophages enhance iNOS mRNA expression for increased NO production. Therefore the ability of inflammatory mediators such as LPS or the Gal-lectin to stimulate TNF-α may be central in activating IFN-γ primed BMM for NO production.

Macrophages are activated by multiple signals delivered in defined sequences. The major priming or initial signal is IFN-γ, and the sequential exposure of IFN-γ + LPS is the best studied model for the induction of TNF-α and NO production by macrophages. In this report, we show that a purified amebic molecule, Gal-lectin, behaved similarly to LPS in activation of IFN-γ-primed BMM as demonstrated by the enhanced iNOS and TNF-α mRNA accumulation, NO production and TNF-α secretion observed in response to the Gal-lectin. Other amebic molecules contained within the soluble *E. histolytica* proteins included in this study did not have any significant effect (inhibitory or enhancing) on primed BMM capabilities. Furthermore, the specificity of Gal-lectin

stimulation was demonstrated by a reduction of its effects in the presence of anti-lectin mAb 1G7, which did not inhibit the activation of BMM in response to LPS. It is not surprising that mAb 1G7 exerted its greatest inhibitory effect at the lowest concentration of Gal-lectin used (100 ng/ml) as higher lectin concentrations may be in excess of the neutralizing antibody which was kept at a constant concentration (1 μg/ml). Previous work using a panel of mAb which recognized non-overlapping epitopes of the cysteine-rich region of the 170 kDa Gal-lectin heavy subunit (32) determined that the recognition site for mAb 1G7 is located between aa 596-818 and is within a domain that mediates both amebic adherence to a target cell and the induction of TNF-α mRNA in naive BMM (24). The results of this study show that this region of the Gal-lectin also is involved in mediating iNOS gene expression and NO production in primed BMM. An unusual but consistent finding of our study was the ability of mAbs 1G7 and R35 to enhance iNOS and TNF-α mRNA expression and TNF-α release in response to IFN-γ + LPS or IFN-γ + Gal-lectin at high concentrations. This may be related to steric hinderance caused by the mAbs rendering the LPS receptors more accessible but this is only speculation. Another possibility is that the combination of the signals received from LPS and the binding of free IgG antibodies (as neither mAb recognizes LPS) to the Fc receptors may cause an overall enhancement of macrophage effector functions.

Activated macrophages release high outputs of TNF-α and NO. Since both these molecules are very important as costimulatory signals or in cytotoxic/cytolytic events we determined if the Gal-lectin can trigger macrophages to be fully activated to kill *E. histolytica* trophozoites. A major finding of our study was that BMM simultaneously treated with IFN-γ + Gal-lectin were activated to kill trophozoites, almost as effectively as IFN-γ + LPS-stimulated BMM. The presence of AG, a nucleophilic hydrazine compound which binds to iNOS isoforms and inhibit NO production (31), greatly reduced the killing activity of the activated BMM. These results demonstrate that

NO is a major effector molecule which mediates the anti-parasitic effect of activated macrophages. As the presence of mAb 1G7 inhibits Gal-lectin (100 ng/ml)-stimulated NO (53%) and TNF-α (61%) production and amebicidal activity (88%) by primed BMM, we propose that the Gal-lectin recognition site for mAb 1G7 (aa 596-818) may be of major importance in activating macrophages.

A common observation during invasive amebiasis is the marked suppression of macrophage effector and accessory cell functions from cells derived from amebic liver abscess (1, 11, 33, 34). Abscess derived macrophages are refractory for killing amebae and NO production (11). In contrast, macrophages isolated from regions distal to the amebic lesions (spleen and peritoneal) are not suppressed and produce NO to kill *E. histolytica* (11). Similarly, BMM pretreated with soluble amebic proteins followed by stimulation with IFN-γ + LPS exhibit a decrease in cytotoxicity against amebae with a corresponding decrease in TNF-α and iNOS mRNA levels as well as TNF-α and NO production (11). Indomethacin, a PGE_2 inhibitor, reversed the observed suppression of TNF-α production, but not NO production nor amebicidal activity. Therefore, amebae or amebic components suppress NO production by macrophages during a primary infection by an unknown mechanism. Similar results are presented in this study whereby soluble amebic proteins in the presence of IFN-γ had no effect on the induction of iNOS and TNF-α mRNA or NO and TNF-α production. Even though soluble amebic proteins contain Gal-lectin (24) there are many other uncharacterized components that have pronounced immunosuppressive effects on naive BMM for TNF-α (27) and iNOS mRNA (11) induction or TNF-α (34) and NO production (11) in response to IFN-γ and/or LPS stimulation. Previous work suggest that abscess derived macrophages are defective in controlling the multiplication and spread of the parasites (1, 11, 33, 34) while the results from this study and those of others (3, 7, 10) indicate that activated macrophages are effective in destroying the parasite. Therefore, in order to be effector cells against

amebae, macrophages must be activated prior to the onset of the suppressive effect mediated by amebic components. The results of this study clearly demonstrate the ability of the native Gal-lectin, together with IFN-γ, to activate macrophages to kill *E. histolytica* trophozoites *in vitro*.

In vitro, the native Gal-lectin has been shown to activate T-cells for IL-2 and IFN-γ production (21, 22). It can be hypothesized that in drug-cured patients or following Gal-lectin immunization, the sensitized T lymphocyte population in the lamina propria will produce IFN-γ which in turn would prime local macrophages at the site of *E. histolytica* infections. Primed macrophages would then be able to respond to a second signal such as the Gal-lectin, and be activated for enhanced TNF-α and NO production for cytotoxicity against the parasite. A long-term goal in our laboratory is to design a subunit vaccine against amebiasis using the Gal-lectin molecule. Our results clearly demonstrate the feasibility of the Gal-lectin, particularly the site between amino acids 596-818, as an effective stimulus for TNF-α and NO production which are vital for host defense against *E. histolytica*. We propose that this region of the Gal-lectin should be further defined to characterize T-cell epitopes for a T_H1 type response which may be required for enhanced production of IFN-γ and subsequent macrophage activation.

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SECTION III: GENERAL DISCUSSION

The most effective preventive measure to eliminate amebiasis consists of improving the living conditions of the population in countries where amebiasis is prevalent (1). This improvement which includes the purification of drinking water and the adequate treatment of sewage would reduce the risk of infection from many intestinal pathogens, including *E. histolytica*. However, as many countries choose to develop economically in ways which disregard the importance of providing clean drinking water for its population, the development of a vaccine against amebiasis appears as a more realistic option. There are factors which favour the control of amebiasis by vaccination: amebiasis is not a vector-borne disease; *E. histolytica* naturally infects only man and does not exist in animal populations as a reservoir; unlike other parasites which have many developmental stages within man, *E. histolytica* has only a motile trophozoite stage and the infective cysts to be of medical concern; amebic surface antigens appear to be antigenically stable; *E. histolytica* infections generate both humoral and cell-mediated immune responses; and finally, the most compelling evidence for the development of a vaccine is that recurrence of amebic liver abscess is rare, suggesting that acquired immunity develops following cure and clearance of invasive infection (2).

When developing vaccines both safety and efficacy must be ensured (3). An ideal vaccine elicits protective immunity and memory so that subsequent exposure to the pathogen will result in elimination of the pathogen and boosted immunity (4) but at the same time should not induce morbidity nor mortality (3). There are advantages of vaccines based on peptide sequences or antigenic molecules as opposed to the use of whole organisms. These advantages include a reduced risk of contaminants and virulence, and an increased availability of vaccine components as peptide-based vaccines contain

specific epitopes recognized by T and B cells yet provide the opportunity for the removal of undesirable sequences such as those that cause immunosuppression or mimic self sequences (4). Therefore, for a successful subunit vaccine, it is important to isolate an immunogenic molecule from *E. histolytica* and to determine its sequences which elicit a protective immune response, specifically the activation of T cells and macrophages which are important for host resistance against amebiasis (reviewed in Chapter II). Up to now, studies testing the efficacy of the Gal-lectin as a vaccine have predominately focused on the functional test of prevention of infectivity, the levels of anti-lectin antibodies and functions of T cells (*ex vivo*) without studying other parameters of the immunogenicity of the Gal-lectin (5-8). Our studies addressed the effect of the Gal-lectin upon macrophages, a cell type which has been ignored in the vaccine studies to date. This work was a primary step to characterize fully the immune responses generated by the Gal-lectin (or portions of the molecule). The objective of this study was initially to characterize the *in vitro* response of macrophages to soluble amebic molecules; secondly, the response of macrophages to a specific native amebic adherence lectin (Gal-lectin) and finally to define the region of the Gal-lectin which elicited the macrophage responses. The principal findings of this research are summarized below.

Activated macrophages are the most important effector cells in protective immunity against *E. histolytica* (reviewed in Chapter II). The work contained in Manuscript III and earlier works (9-13) demonstrated that human monocytes and murine macrophages activated with IFN-γ + LPS or TNF-α kill amebic trophozoites *in vitro*. However, macrophages isolated from amebic liver granulomas are not effective in controlling the spread of the parasite, are refractory to IFN-γ + LPS activation to kill the amebae, and have diminished ability to produce nitric oxide (13), TNF-α (14) and Ia antigen expression (15). A similar down-regulation of macrophage functions was observed *in vitro* following pretreatment of naive macrophages with soluble amebic

proteins (13-15). We hypothesized that the *E. histolytica* proteins can modulate macrophage functions and therefore sought to determine the mechanisms involved. The rationale for this is that *in vivo*, amebic granuloma macrophages at the margin of the lesions are continually exposed to excretory and secretory amebic components and whole cell lysates which provides ample opportunity for amebic molecules to modulate macrophage functions. The results in Manuscript I demonstrated that soluble *E. histolytica* proteins can stimulate a brief and transient increase of TNF-α and c-*fos* mRNA levels through PKC signal transduction in naive macrophages. However, *E. histolytica*-induced TNF-α and c-*fos* mRNAs were rapidly degraded as compared to LPS stimulated mRNA expression. This rapid degradation in *E. histolytica* stimulated macrophages could be a novel mechanism invoked by the parasite to suppress TNF-α production and thus, macrophage activation. Furthermore, pretreatment with soluble *E. histolytica* proteins reduced TNF-α mRNA expression by macrophages in response to known stimulators (LPS or IFN-γ + LPS) demonstrating that *E. histolytica* modulates macrophages at the level of transcription. The repressor factors involved in the diminished transcription of genes in macrophages stimulated with *E. histolytica* will be an interesting area to pursue in further studies.

As soluble *E. histolytica* proteins is a mixture of many components which can either prime or suppress macrophage functions, we focused our studies on a single native amebic molecule. We selected the Gal-lectin as it mediates the adherence of *E. histolytica* to target cells which is an absolute prerequisite for amebic cytotoxicity (16, 17). Besides its importance in pathogenesis, the Gal-lectin stimulated sensitized T-lymphocytes isolated from humans and gerbils to proliferate and to produce IL-2 and IFN-γ (8, 18, 19). More importantly, the Gal-lectin, used either as a native molecule or as a recombinant fusion protein, successfully protected gerbils from developing amebic liver abscesses when challenged with live trophozoites (5-8). The results in Manuscript

II demonstrate that the Gal-lectin can stimulate naive murine macrophages for stable TNF-α mRNA expression and protein production. TNF-α production is a characteristic of an activated macrophage and recently, we have shown the contribution of TNF-α produced by activated macrophages for enhanced inducible nitric oxide synthase mRNA expression in macrophages for nitric oxide-dependent cytotoxicity against *E. histolytica* trophozoites (12). TNF-α produced by macrophages, therefore, is central for cell-mediated immunity against amebiasis. Despite its capacity to stimulate TNF-α production, the Gal-lectin failed to activate naive macrophages for NO production or amebicidal activity. However, it is important to remember that macrophage activation proceeds through several functional stages beginning with a responsive state then to a primed state and finally to a fully activated state (reviewed in Chapter III). In the circulation, IFN-γ is the primary macrophage-priming factor. IFN-γ activates numerous macrophage functions including cytotoxicity, antigen processing and presentation to lymphocytes, and acts synergistically with other agents to induce macrophage production of TNF-α and NO. We chose to determine the effect of the Gal-lectin on IFN-γ primed macrophages as it was previously demonstrated that the Gal-lectin stimulates IFN-γ production by sensitized T-cells *ex vivo* (8, 18). In Manuscript III, we have shown that the Gal-lectin, in concentrations as low as 100 ng/ml, activated IFN-γ primed macrophages for enhanced TNF-α and iNOS mRNA expression and TNF-α and NO production. Furthermore, macrophages treated simultaneously with IFN-γ and Gal-lectin were activated to kill amebic trophozoites *in vitro* (Manuscript III). Collectively, the results from manuscripts II and III demonstrate that the Gal-lectin stimulates macrophages. However, observations from Manuscript III indicate that activated T-cells of T-helper 1 (T_H1) lineage are also necessary to produce IFN-γ which is required to prime macrophages for NO production and amebicidal activity.

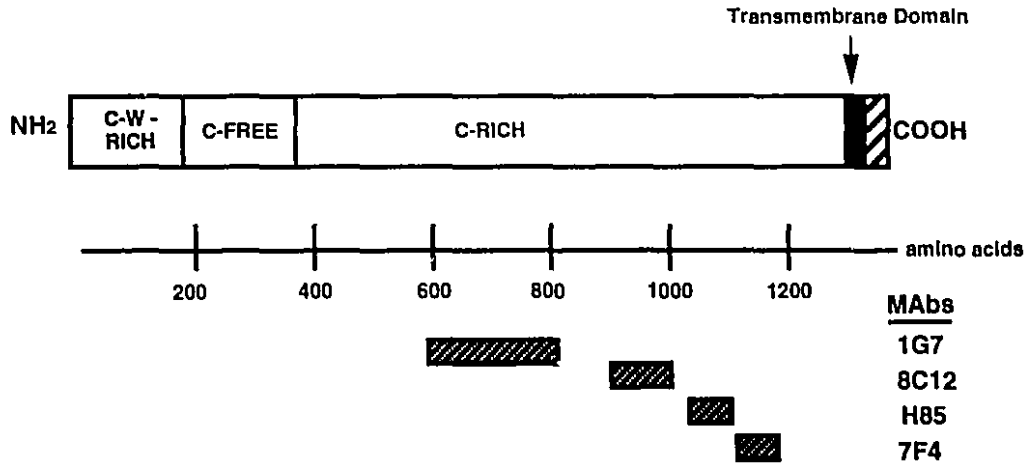

Figure 1. The location of the epitopes recognized by 170 kDa subunit-specific mAbs. The location of the various epitopes are depicted by the solid boxes below the domain structure diagram. C and W refer to cysteine and tryptophan respectively. Figure has been modified from Mann, B. *et al.* 1993.

The determination of the sequences of the Gal-lectin that mediated the observed effects was of paramount importance to define the immunostimulatory region of the Gal-lectin. Using a panel of anti-lectin monoclonal antibodies whose recognition sites were previously mapped to non-overlapping epitopes of the 170 kDa heavy subunit (Fig. 1)(20), we attempted to inhibit functions of the Gal-lectin, specifically amebic adherence to a target cell and macrophage modulation. Three mAbs (1G7, H85 and 8C12) inhibited both amebic adherence and TNF-α mRNA induction by macrophages (Manuscript II) while one mAb tested (1G7) inhibited TNF-α and iNOS mRNA expression and TNF-α and NO production by IFN-γ primed macrophages (Manuscript III). mAB 1G7 also inhibited the activation of macrophages to kill trophozoites *in vitro* following treatment with IFN-γ + Gal-lectin (Manuscript III). These results suggest that the region between aa 596-1082 of the 170 kDa heavy subunit of the Gal-lectin mediates both amebic adherence and TNF-α mRNA induction in macrophages and that the region between aa 596-818 participates in activating primed macrophages. Previously, two independent vaccine

studies using recombinant fusion proteins containing the regions between aa 758-1134 and aa 649-1202 of the 170 kDa subunit yielded 71 and 81% efficacy, respectively, to protect gerbils upon challenge (6,7). Both trial recombinant vaccines contained portions of the region of the Gal-lectin which we had determined to mediate an immune response in macrophages. However, the region between aa 895-998 (20) contains the recognition sites for two adherence enhancing anti-Gal-lectin mAb (3F4 and 8A3)(19), suggesting that this region of the Gal-lectin may not necessarily be protective. In future vaccine studies this region of the Gal-lectin should be removed as it may reduce the efficacy of the immune stimulatory properties of the Gal-lectin. Results from Manuscript III suggest that aa 596-818 are important in activating macrophages. We propose that this region of the 170 kDa subunit, which is not recognized by adherence enhancing mAbs, warrants further study to determine the T-cell epitopes which stimulate a T_H1 response as our results demonstrate the importance of IFN-γ production for immune cell activation and host resistance. This region of the 170 kDa subunit is possibly the best choice for a subunit vaccine candidate. However, it is clear that more work remains to be done before using the Gal-lectin as a subunit vaccine as our studies looked only at the effect of the native Gal-lectin and did not consider the effects of a recombinant fusion product or the effect of a carrier of the subunit vaccine. Furthermore, it is important to note that all the work contained within this thesis was performed *in vitro* and would need to be repeated *in vivo*. Nevertheless, this research work was necessary as macrophages play a critical role in host defence yet their responses were neglected by previous studies.

Future studies should use attenuated strains of *Salmonella* which express portions of the Gal-lectin to induce memory T-cells (T_H1) and an IFN-γ-biased response. Live attenuated vaccines usually induce a better level of protection than purified or recombinant immunogens or non-living organisms (22) as live vaccines have greater antigen persistence, induce both mucosal and systemic immune responses and are able to

target macrophages in a form that induces a protective immune response which is necessary for host resistance against amebiasis.

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