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Lipid microdomains are involved in adhesion of Entamoeba histolytica trophozoites to host extracellular matrix components

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LIPID MICRODOMAINS ARE INVOLVED IN ADHESION OF *ENTAMOEBA HISTOLYTICA* TROPHOZOITES TO HOST EXTRACELLULAR MATRIX COMPONENTS

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ABSTRACT

Entamoeba histolytica is a parasitic human protozoan that infects 500,000,000 people worldwide annually. In the course of the parasite's life cycle, motile trophozoites breach the colonic mucosa, invade through the epithelial layer and extracellular matrix (ECM) and occasionally disseminate through portal blood vessels to distant organs. Membrane rafts are small heterogeneous, highly dynamic, sterol- and sphingolipid- enriched domains whose functional significance entails compartmentalization of cellular processes and regulation of cellular signaling. Recent studies reveal the physiological role of membrane rafts in adhesion to host epithelium in *E. histolytica*. In the current study we examined the role of lipid rafts in adhesion of trophozoites to host ECM components, collagen and fibronectin. A high throughput fluorescence based assay was developed to assess parasitic adhesion to commercial collagen type I- and fibronectin-coated microtiter plates. Disruption of membrane rafts by treatment with a cholesterol extracting agent, methyl-beta-cyclodextrin (MβCD), resulted in inhibition of adhesion to ECM. Replenishment of cholesterol by treatment with a lipoprotein-cholesterol concentrate (LCC) restored adhesion. Confocal microscopy, using fluorescent lipid analogs, revealed enrichment of lipids at the parasite-ECM interface. The galactose inhibitable Gal/GalNAc lectin is a glycoprotein on *E. histolytica* that is a known resident of lipid rafts and mediates adhesion to host cells. Adhesion to collagen was observed to decline in

the presence of galactose, suggesting a role for the Gal/GalNAc lectin as a putative receptor mediating adhesion to collagen. On the other hand, adhesion to fibronectin was not impaired by galactose, suggesting that the Gal/GalNAc lectin is not involved in adhesion of *E. histolytica* to fibronectin. This study has offered new insight into the molecular mechanisms of adhesion, which is important to the pathogenesis of amoebiasis. Such insight may lead to the development of innovative therapeutic modalities and vaccines.

DEDICATION

To my beloved grandmother, Late Smt. Sharda Devi Mittal and my dear grandfather Shri K.K. Mittal. The morals and family values that you have instilled in your children and grandchildren have cemented the strong bond that holds us all together. I cherish with fondness the love and affection that you have bestowed upon me. I hope you can look upon your lives with a sense of satisfaction, and know that with each passing day, I miss you more.

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CHAPTER 1

LITERATURE REVIEW

I. Introduction

Epidemiological significance

Entamoeba histolytica is a human intestinal pathogen that causes approximately 100,000 deaths worldwide annually [1]. Interestingly, the origin of its name ("histolytica") bears reference to its "tissue destroying ability" [reviewed in 2]. In terms of mortality attributed to protozoan parasites, *E. histolytica* ranks second worldwide, next only to malaria, thus emphasizing the epidemiological significance of this infectious agent [1]. Previously, based on results of microscopic stool analysis, *E. histolytica* was believed to infect 500 million people worldwide. However, advances in diagnostic techniques have revealed the existence of two morphologically identical but antigenically distinct strains, *Entamoeba histolytica* and *Entamoeba dispar.* These strains bear biochemical and genetic differences, and can be antigenically distinguished using PCR-based commercially available kits. It is now believed that *E. histolytica* is primarily a pathogenic strain, whereas *E. dispar* is a non-pathogenic commensal [reviewed in 3].

E. histolytica is transmitted through the fecal-oral route, and there is higher disease prevalence in areas with inadequate sanitation and environmental hygiene. In industrialized countries travelers, immigrants, institutionalize populations and homo-

sexual men constitute the high risk group for contracting the disease [4]. *E. histolytica* has emerged as an important opportunistic pathogen in chronically immunosuppressed patients, such as people suffering from HIV-AIDS. Such patients are also at a higher risk of developing invasive amoebiasis, in comparison to immune-competent subjects [5].

Given the ease of transmission by the fecal-oral route and the morbidity associated with amoebic dysentery, it is no surprise that *E. histolytica* has been classified as a category B bioterrorism agent by the National Institutes of Health. That the parasite can be manipulated genetically and that amoebiasis is difficult to diagnose supports its classification as an agent of biowarfare. Therefore, there is elevated priority to understand pathogenesis; such insight may lead to new methods of disease prevention, detection and treatment.

Life cycle and pathogenesis

E. histolytica is a unicellular eukaryotic protozoon that is transmitted by the fecal-oral route. It exhibits a simple life cycle consisting of two stages, namely the cyst and the trophozoite. *E. histolytica* cysts are round, quadrinucleated and measure 10-15 μm in diameter. Ingestion of infective cysts occurs via contaminated food and water. So far, humans and a few primates are the only known natural hosts for *E. histolytica*. The cysts are resistant to the acidic environment of the gastric lumen, and excystation occurs in the terminal ileum and colon. After excystation, both the cytoplasm and nuclei divide to produce eight metacystic, motile trophozoites, which measure 10-50 μm in diameter. In

most cases, trophozoites re-encyst within the lumen of the colon and the cysts are passed in stool, thus completing the life cycle of the parasite [6].

In some cases, the colonic lumen may become colonized by trophozoites, a process that involves interaction of trophozoites with the protective layer of mucin that lines the intestinal epithelium and forms the body's first line of defense [7]. This interaction involves adhesion, degradation and subsequent invasion of mucin, which brings the parasite in contact with submucosal epithelium. During such invasion, trophozoites are driven by nourishment derived from intestinal bacteria and food particulates [6]. Amoebapore, a polypeptide that disrupts bacterial and host cell membranes, and cysteine proteases, a group of enzymes that degrade host cells and ECM, are secreted extracellularly by trophozoites at this stage and are important virulence factors that regulate invasion [2].

Destruction of intestinal epithelium and degradation of extracellular matrix (ECM) that surrounds epithelial cells results in formation of flask-shaped amoebic ulcers [8]. These pathological changes are manifested in the form of watery diarrhea, dysentery, tenesmus and colitic pain in the abdomen. Highly invasive trophozoites encounter the vascular tissue in the vicinity of the ulcers, and may disseminate through the blood stream to other organs, such as liver, lungs and brain [2]. Patients of amoebic liver abscess present with pain in abdomen, pyrexia, weight loss and fatigue. In rare instances, other complications may include cutaneous amoebic ulcers, subphrenic abscess, pericarditis and peritonitis [9].

Currently, medical management with nitroimidazoles is the first line of treatment of amoebiasis. However, the rate of luminal eradication of cysts with this drug is not very high. There has also been emergence of drug resistance that has further compounded the problem of disease eradication [reviewed in 2]. A comprehensive understanding of the molecular mechanisms of pathogenesis can thus contribute significantly to the development of potential drug targets and novel approaches to vaccine development.

II. Physiological role of adhesion

Adhesion is a critical step in the pathogenesis of amoebiasis. *E. histolytica* adhere to bacteria in the colonic lumen, a process that provides nourishment and sustains the parasite [6]. Association of *Escherichia coli* with *E. histolytica* trophozoites *in vitro* has been shown to enhance host cell destruction [10]. Additionally, studies indicate that exposure to *E. coli* induces transcription of various genes such as protein kinase, ABC transporter, Rab family GTPases and hsp 90, all of which may enhance the phagocytic capacity of *E. histolytica* [11]. Therefore, adhesion to bacterial flora of the intestine may also modulate parasitic virulence. Subsequent stages of colonization and invasion of the human body involve adhesion of the parasite to mucin, intestinal epithelium and ECM components.

Mucins are glycoproteins possessing a significant number of O-linked glycan modifications. This mucous gel layer is the body's first line of defense against infection. Colonization in the intestine is initiated by adherence of trophozoites to host glycoconjugates via specific, yet poorly understood, receptors [12]. Evidence suggests

that the association between *E. histolytica* trophozoites and colonic mucins is mediated by a galactose and N-acetyl-D-galactosamine inhibitable lectin on the amoebic surface, known as the Gal/GalNAc lectin [7]. It has been shown that interaction with mucins may trigger signaling pathways in *E. histolytica*, especially those that regulate encystation [13]. Evidence that the Gal/GalNAc lectin is involved in adhesion to mucin includes the observation that galactose and N-acetyl-D-galactosamine residues of mucins specifically inhibit binding of the amebic 170 kDa heavy subunit of the Gal/GalNAc lectin to target cells [7]. Additionally, there is also evidence that the interaction of trophozoites with mucins decrease cytolysis of host epithelium *in vitro* [7].

Once the mucin barrier is breached, *E. histolytica* trophozoites can bind to host cells. It has been previously reported that *E. histolytica* kills target cells in a contact-dependant fashion [14]. The host target cells studied include a number of cell types of epithelial origin as well as erythrocytes that are encountered during host tissue destruction. The latter, which may also be taken up by phagocytosis, may serve as a source of nourishment for invasive trophozoites [15]. These findings indicate that adherence of trophozoites to target cells is an important step involved in disease pathogenesis. Subsequent to cytolysis of epithelial cells, invading trophozoites encounter the ECM. Collagen and fibronectin are important ECM components that have been studied in the context of *E. histolytica*-host interaction [16, 17].

Several lines of evidence suggest that interaction of *E. histolytica* with ECM components in the invasive stage of amoebiasis may be likened to focal adhesions of higher

eukaryotes [16, 17]. Focal adhesions are complex, dynamic supramolecular aggregates containing integrins, which are α/β heterodimeric proteins occurring at the sites of cellular attachment to ECM [18]. A more detailed discussion of proteins of the integrin family is provided below. In mammalian cells, focal adhesions are characterized by interaction of integrins with cytoplasmic proteins and cytoskeletal elements, thus acting as the mechanical link between ECM and the cytoplasm [reviewed in 18]. Interestingly, integrins can also propagate ECM-induced signaling. It is believed that the interaction of *E. histolytica* with host ECM components can alter signal transduction pathways and enhance parasitic virulence [16, 19, 20]. For example, exposure of *E. histolytica* trophozoites to collagen induces actin accumulation in adhesion plates and phosphorylation of tyrosine residues on the *E. histolytica* homolog of $pp125^{FAK}$ [17, 21]. In mammalian cells, adhesion-induced focal adhesion kinase (FAK) activation has been described as a hallmark of integrin-mediated signaling. $pp125^{FAK}$ is a non receptor cytosolic protein that localizes to focal adhesion plaques and is a substrate for tyrosine kinase phosphorylation [21]. Exposure of *E. histolytica* to collagen also stimulates $pp125^{FAK}$ association with paxillin and Src (pp^{60src}), which may result in increased DNA binding of the transcription factor AP-1 [22]. Collagen also induces phosphorylation of $p42^{MAPK}$, which may propagate a phosphorylation-based signal from the plasma membrane to the nucleus [21]. In addition, exposure of trophozoites to collagen has been shown to increase DNA binding of other *E. histolytica* transcription factors such as STAT1 and STAT3 [19]. This, in turn, may regulate changes in gene expression. In support of this, collagen exposure results in increased expression of amoebapore and a cysteine protease, two proteins that are secreted by *E. histolytica* [20]. Thus, the

upregulation of their expression upon exposure of trophozoites to collagen emphasizes the significance of trophozoite-ECM interactions in host invasion.

Likewise, binding of *E. histolytica* trophozoites to fibronectin induces phosphorylation of $pp125^{FAK}$, association of an integrin like receptor with paxillin, and activation of protein kinase A (PKA) [23, 24], a protein involved in G-protein coupled receptor signaling. Additionally, exposure to fibronectin induces reorganization of actin and its redistribution to the sites of adhesion [16, 25]. It is thus surmised that the interaction of *E. histolytica* trophozoites with ligands on target cell surface, and on extracellular host components, may trigger signaling pathways within the trophozoites, in addition to intracellular cytoskeletal rearrangements. Thus, a better understanding of this interaction at the molecular level can provide further insight into determinants of invasion and disease pathogenesis.

III. Molecular components involved in adhesion

Several adhesion molecules of *E. histolytica* that are involved in amoebic adhesion to host components have been described [reviewed in 2]. These include a cysteine protease (EhCPADH112), L220, serine-rich *E. histolytica* protein (SREHP), the Gal/ GalNAc lectin, and several integrin-like receptors.

The EhCPADH112 is a transmembrane protein that localizes to the cysteine protease-adhesin complex in the plasma membrane and phagosomes of trophozoites. Though the ligand specificity of this adhesion molecule is still under investigation, *in vitro*

experiments indicate that adhesion to host cells is inhibited in the presence of antibodies to this protein, thereby establishing its role in the process of adhesion [26, 27]. The L220 is a 220 kDa lectin-like protein on the plasma membrane involved in binding to host cells. The SREHP is another surface protein implicated in adhesion to host components. While antibodies to SREHP as well as L220 are known to inhibit adhesion to host cells, their exact ligand binding properties are still unclear [28-30]. The adhesion proteins that are most relevant to this study are the Gal/GalNAc lectin, and integrin-like proteins, both of which are described subsequently.

Gal/GalNAc lectin: trophozoite interaction with host cell and mucin

The significance of adhesion in the pathogenesis of amoebiasis sparked interest in the scientific community as far back as the early 1980s. Studies examining the effects of carbohydrates on adhesion of *E. histolytica* to Chinese hamster ovary (CHO) cells and human erythrocytes (RBC) revealed that GalNAc inhibits adhesion to host cells by binding a receptor on the amoebic surface [14]. It was also revealed from this study that adherence is required prior to target cell lysis, the latter being an event which is concomitantly inhibited in the presence of GalNAc. Thus, it was evident that *E. histolytica* trophozoites adhere to host cell surface via a specific amoebic receptor that possesses affinity for GalNAc. In 1985, the first report describing a soluble GalNAc-inhibitable lectin in *E. histolytica* emerged. A 43-67 kDa Gal/GalNAc lectin, as it was named, was found to agglutinate host epithelial cells, erythrocytes, and polymorphonuclear neutrophils [31].

Subsequently, the subunit structure of Gal/GalNAc lectin was discovered. The Gal/GalNAc lectin is composed of a 170 kDa transmembrane heavy chain subunit (Hgl) linked via a disulphide bond to a 31-35 kDa glycosylphosphatidylinositol (GPI)-anchored light subunit (Lgl). These subunits are non-covalently associated with a 150 kDa intermediate subunit (Igl) [reviewed in 2]. The Hgl subunit is comprised of a large cysteine-rich extracellular domain, a single transmembrane domain, and a 41 amino acid cytoplasmic domain [32]. Hgl exhibits some homology in its C terminus with β2 and β7 mammalian integrins [33, 34]. The Lgl and Igl subunits lack cytoplasmic domains [35].

That the Gal/GalNAc lectin is a cell surface protein has been confirmed by indirect immunofluorescence [36]. It is believed to be part of a signal-associated complex [37], and proteomic studies have revealed the association of its subunits with cytoskeletal and signaling proteins [35]. Monoclonal antibodies specific for the 170 kDa receptor have been demonstrated to inhibit binding of trophozoites to mucin [7]. Additionally, incubation of trophozoites onto host epithelial cells, in the presence of antibodies directed against the heavy subunit (Hgl) has shown nearly 100% inhibition of adhesion, implicating Hgl as a primary contributor involved in adhesion to host cells [38]. Therefore, it is likely that the Hgl is involved in adhesion to multiple host components at various stages of invasion. Characterization of binding affinities of glycoconjugates with terminal Gal and Gal/GalNAc residues has indicated that carbohydrate ligands with multiple Gal/GalNAc residues (i.e., multivalent) are the most potent inhibitors of trophozoite adhesion [12].

Interestingly, the Gal/GalNAc lectin is strongly immunogenic, and has been shown to elicit an anti-lectin IgA response in rats. Moreover, isolated IgA purified from immunized animals possesses inhibitory activity against adhesion *in vitro* [39]. More recently, clinical trials in Bangladesh, an amoebiasis-endemic country, have indicated that a mucosal IgA anti-lectin antibody response in humans confers immunity against *E. histolytica* colonization [40]. Thus, the Gal/GalNAc is now recognized as a prime target for subunit vaccine development [41].

Integrin-like receptors: trophozoite interaction with ECM

Integrins are transmembrane adhesion proteins comprised of α/β heterodimers, which link the ECM components to cell cytoskeleton [42]. When cells come in contact with extracellular substrates, integrin molecules, that are engaged by ECM ligands, induce various intracellular signaling pathways through outside to inside signaling. Concurrently, focal adhesions are formed at the cell-ECM interface that connect ECM, integrins, cytoskeletal adaptor proteins (talin, vinculin, actin), as well as signaling proteins, resulting in inside to outside signaling [42]. The ligand specificity during cell-ECM binding is determined by α/β association of integrin heterodimers [18]. So far, in *E. histolytica*, only proteins with homology to β subunits of integrins have been isolated [24, 43].

Interaction of trophozoites with fibronectin has been shown to induce formation of focal adhesion-like structures, which recruit polymerized actin [16, 25].This interaction is postulated to be mediated by a fibronectin receptor (β1*Eh*FNR) which, upon adhesion to

fibronectin, assembles a supramolecular signaling complex that induces tyrosine phosphorylation. It has been shown that this multimolecular complex is composed of the β1*Eh*FNR, FAK, paxillin and vinculin [24]. Characterization of the β1*Eh*FNR has revealed that it is a 140 kDa protein localized to the cell surface [44]. This receptor is recognized by a human anti-β_1 integrin 3C10 monoclonal antibody in immunoblot assays [45]. This antibody has been found to significantly inhibit adhesion of trophozoites to fibronectin and collagen, and partially to another ECM component, laminin, thereby implicating a role for β1*Eh*FNR in adhesion to each of these extracellular substrates. In further support of this, earlier studies have shown that a β-integrin-like molecule colocalizes with actin as well as collagen in trophozoites exposed to collagen [17]. A recent study demonstrated mobilization of the receptor from internal vesicles to the plasma membrane on stimulation with fibronectin [45]. Perhaps the most interesting discovery in this regard has been that the amino acid sequence for the β1*Eh*FNR shares 99% and 96% homology with the genes encoding for Igl2 and Igl1 (intermediate subunit of the Gal/GalNAc lectin), respectively, thus providing a link between the two widely studied amoebic adhesion receptors for host cells and ECM [45].

A recent study has provided evidence for a second, distinct receptor that shares a homologous epitope with neutrophilic β2 integrin [43]. This receptor is distinct from the Gal/GalNAc lectin, and anti-β2 integrin antibody has been found to inhibit adhesion of *E. histolytica* trophozoites to TNF-*α*-activated ICAM-expressing cells [43]. ICAM is the traditional legend for β_2 integrins. This discovery is exciting as it has opened avenues for

future research into the potential role of this molecule in adhesion, as well, as identification of extracellular ligands for this receptor.

Purification of membrane proteins that interact with collagen at 37^{o}C in *E. histolytica* has revealed the existence of seven plasma membrane proteins ranging from 51 kDa to 220 kDa. These proteins are hydrophilic, and thus putatively reside on the extracellular surface of the pathogen [46]. Further characterization of interaction of these proteins with collagen may identify a putative receptor for collagen.

Lipid rafts and their role in adhesion

Membrane rafts are defined as highly dynamic sterol- and sphingolipid-enriched domains that compartmentalize cellular processes [47]. Rafts are 10-200 nm platforms that spatially and temporally regulate physiological events. They are resistant to non-ionic detergent lysis at 4^{o}C and hence referred to as detergent-resistant membranes (DRMs). Lipid rafts have also been shown to possess transmembrane proteins recognized for trafficking. Some proteins constitutively reside in rafts, such as the GPI-anchored proteins, while other proteins may accumulate within rafts upon oligomerization or engagement with ligands [48]. A constitutive raft protein, the monosialoganglioside and glycosphingolipid, GM1, has been frequently utilized as a raft marker [49].

The biological role of lipid rafts has been the subject of numerous studies, which have implicated them in cellular processes like membrane sorting and trafficking, signal transduction and cell polarization [50]. There is a mounting body of evidence suggesting

the involvement of lipid rafts in multiple stages of host-pathogen interactions, including adhesion, internalization, phagosome maturation and lysosomal fusion, intracellular signaling, apoptotic induction and cytokine secretion [51]. Recent evidence suggests that lipid rafts in the plasma membrane of *E. histolytica* play an important role in endocytosis, secretion and adhesion of the parasite to host cells [52]. The existence and physiological relevance of these rafts in *E. histolytica* has been shown through a variety of methods. For example, fluorescence microscopy using a fluorescent lipid analog, dialkyindocarbocyanine ($DiIC_{16}$), has revealed raft enrichment in plasma membranes as well as intracellular structures. Moreover, depletion of cholesterol by treatment of cells with methyl-beta-cyclo-dextrin (MβCD), a reagent that encapsulates cholesterol in its hydrophobic core, has been found to abolish $DiIC_{16}$ staining in the plasma membrane. Raft disruption using MβCD has also been found to significantly inhibit adhesion of *E. histolytica* to host epithelial cells. Finally, using sucrose density centrifugation, rafts have been purified from *E. histolytica* membranes and have been found to be enriched with the Gal/GalNAc lectin [52].

In higher eukaryotes, lipid rafts serve as signaling platforms in which integrins, as well as other adhesion/signaling molecules, may reside or accumulate in a signal-dependent fashion [53, 54]. Various cell surface receptors involved in signal transduction have been reported to associate with lipid rafts, including integrins [55]. Previous studies indicate that the amoebic adhesion molecule Gal/GalNAc lectin is localized to rafts, thereby supporting the role of membrane microdomains in adhesion of *E. histolytica* [52]. It has been established that lipid rafts also play an important role in adhesion of neural

precursor cells to extracellular matrix [56]. Furthermore, Huang *et al*. [57] recently demonstrated that disruption of lipid rafts in human cancer cell lines inhibits cellular adhesion to fibronectin, collagen and laminin, thereby affecting a crucial step in tumor invasion and metastasis. However, so far, it is unclear whether lipid rafts are involved in adhesion of *E. histolytica* to host ECM components and mucin.

Cholesterol has been described as the dynamic glue that maintains raft assembly, as it has a higher affinity for raft sphingolipids than for unsaturated phospholipids [50]. Being a major constituent of eukaryotic membranes, it is conceivable that the membrane cholesterol content might impact lipid raft assembly and function. Previous research has indicated that acute depletion of cholesterol content of macrophages significantly decreases their interaction with *Leishmania donovani* promastigotes, by disruption of lipid rafts [58]. On the other hand, an increase in membrane cholesterol content in fibroblasts transformed with polyoma virus (PyF) has been shown to restore the transformation-related loss of adhesivity in these cells [59]. Unpublished data from our laboratory indicate that treatment with lipoprotein-cholesterol may enhance virulence functions of *E. histolytica* like erythrophagocytosis and host cell cytolysis in a dose dependant manner, presumably in the context of lipid rafts. Lujan and Diamond [60] have previously indicated that *E. histolytica* may possess a *de novo* route of cholesterol synthesis. However, subsequent studies have demonstrated the requirement of lipoproteins rather than cholesterol, in the absence of which trophozoites cannot be cultivated [61]. This important observation suggests that *E. histolytica* may not possess functional machinery to synthesize cholesterol, and may rely on extracellular sources for

the same. Thus, host cholesterol may be necessary for metabolism and survival of the parasite.

Epidemiological data has demonstrated that lipid parameters might have an impact on the outcome of parasitic infections. In a study conducted amongst patients of amoebiasis in India, it was found that patients with non invasive amoebiasis (cyst passers) had lower serum cholesterol levels in comparison to patients with invasive amoebiasis (amoebic liver abscess) [62]. Interestingly, the most common site for manifestation of extraintestinal amoebiasis is the liver, which is also the primary site for cholesterol synthesis in the human body [63]. Laboratory evidence indicates that lipoprotein enrichment can support *E. histolytica* growth in serum free media [64]. Since trophozoites are unable to utilize free cholesterol *in vitro*, it is possible that *in vivo*, cholesterol-enriched lipoprotein particles in the colonic lumen, tissue and/or serum act as a source of cholesterol in successive stages of invasive disease [63]. In support of this notion, it has been reported that a gradual decline in the ability of trophozoites to induce hepatic abscess in hamsters occurs on prolonged growth in culture. More importantly, this decrease in virulence is reversed on passage through hamster liver or through growth in cholesterol-rich media [65]. Therefore, host cholesterol levels might regulate parasitic virulence, but the exact mechanisms by which host lipids might influence pathogenecity are still unclear. Taken together, these observations suggest that lipid rafts have a well characterized role in cellular adhesion, and alterations in membrane cholesterol levels can impact adhesivity of cells.

IV. Implications and scope for the future

Great progress made in the past few years has advanced our understanding of the determinants of parasitic invasion in *E. histolytica*. These investigations have revealed fresh insight into the molecular mechanisms of disease, providing an impetus for vaccine development and therapeutic targets. Specifically, the discovery of lipid rafts in *E. histolytica* has been an important breakthrough [52]. The fact that the highly antigenic Gal/GalNAc lectins, that are currently being used to develop new vaccines, localize to lipid rafts, is a significant discovery. Therefore, an understanding of lipid raft function and components is necessary to fully understanding *E. histolytica* virulence.

Despite the discovery of a specific receptor for *E. histolytica*-fibronectin interaction [45], there is still uncertainty as to whether this receptor is lipid raft associated, or raft-independent. Since it shares greater than 96% homology with Igl, which is a raft-resident glycoprotein, it is possible that the *Eh*FNR may reside in rafts. There is also the possibility that multiple receptors may be involved in adhesion to fibronectin, some of which may be raft-associated. As for collagen, there is still considerable work that needs to be done before a comprehensive understanding of the parasite's interaction with collagen can be attained. At the same time, it is essential that the mechanisms involved in adhesion to the first line of immunological defense mucin, are explored further. Although the Gal/GalNAc lectin is a well-established receptor for mucin, there is no concrete evidence implicating the role of lipid rafts in this interaction. An additional question that remains unanswered is whether there is an α-subunit homolog in *E. histolytica* integrin-

like proteins. Although it is possible that functional, monomeric β subunits are expressed, there is still insufficient evidence to rule out the presence of an α-subunit homolog.

This work is an investigation into the role of lipid rafts in adhesion to ECM components, collagen and fibronectin. This study represents an attempt to answer some of the questions outlined above, in order to enhance our understanding of molecular mechanisms of amoebiasis. It is hoped that advancements in cell biology will enable the prevention and eradication of this infectious agent.

V. Summary

Lipid rafts are heterogeneous, highly dynamic sterol- and sphingolipid-enriched domains that spatially and temporally regulate physiological events. In *E. histolytica*, these cholesterol-rich membranes play an important role in endocytosis, secretion and adhesion of the parasite to host cells. To date, only one adhesion molecule, that is, the Gal/GalNAc lectin, has been localized to rafts. In this study, we will investigate the role of lipid rafts in adhesion of *E. histolytica* trophozoites to host ECM. More specifically, we will examine the effects of cholesterol depletion as well as lipoprotein supplementation on trophozoite adhesion to host ECM components, collagen and fibronectin. Further, we will attempt to determine if the Gal/GalNAc lectin is the putative receptor within the rafts that mediates adhesion to ECM. Thus, the specific aims for this study are:

1. To develop an assay to assess adhesion of *E. histolytica* trophozoites to ECM components collagen and fibronectin.

2. To determine if lipid rafts are involved in adhesion to collagen and fibronectin.
3. To determine whether the adhesion molecule Gal/GalNAc lectin, is the putative receptor mediating trophozoite adhesion to ECM.

This is the first report that provides evidence suggesting involvement of lipid rafts in adhesion of *E. histolytica* trophozoites to collagen and to fibronectin and the role of Gal/GalNAc lectin in adhesion to collagen.

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CHAPTER 2

LIPID MICRODOMAINS ARE INVOLVED IN ADHESION OF *ENTAMOEBA HISTOLYTICA* TROPHOZOITES TO HOST EXTRACELLULAR MATRIX COMPONENTS.

I. Abstract

Entamoeba histolytica is a parasitic human protozoan that afflicts 50,000,000 people worldwide annually. In the course of the parasite's life cycle, motile trophozoites breach the colonic mucosa, invade through the epithelial layer and extracellular matrix (ECM) and occasionally disseminate through portal blood vessels to distant organs. Membrane rafts are small heterogeneous, highly dynamic, sterol- and sphingolipid- enriched domains whose functional significance entails compartmentalization of cellular processes and regulation of cellular signaling. Recent studies reveal the physiological role of membrane rafts in adhesion to host epithelium in *E. histolytica*. In the current study we examined the role of lipid rafts in adhesion of trophozoites to host ECM components, collagen and fibronectin. A high throughput fluorescence based assay was developed to assess parasitic adhesion to commercial collagen type I- and fibronectin-coated microtiter plates. Disruption of membrane rafts by treatment with a cholesterol extracting agent, methyl-beta-cyclodextrin (MβCD), resulted in inhibition of adhesion to ECM. Replenishment of cholesterol by treatment with a lipoprotein-cholesterol concentrate (LCC) restored the inhibition of adhesion. Confocal microscopy, using fluorescent lipid

analogs, revealed enrichment of lipids at the parasite-ECM interface. The galactose inhibitable Gal/GalNAc lectin is a glycoprotein on *E. histolytica* that is a known resident of lipid rafts and mediates adhesion to host cells. Adhesion to collagen was observed to decline in the presence of galactose, suggesting a role for the Gal/GalNAc lectin as a putative receptor mediating adhesion to collagen. On the other hand, adhesion to fibronectin was not impaired by galactose, suggesting that the Gal/GalNAc lectin is not involved in adhesion of *E. histolytica* to fibronectin. This study has offered new insight into the molecular mechanisms of adhesion, which is important to the pathogenesis of amoebiasis. Such insight may lead to the development of innovative therapeutic modalities and vaccines.

II. Introduction

Entamoeba histolytica is a human intestinal pathogen that ranks second as a cause of morbidity and mortality due to parasitic infections worldwide [1]. Transmitted by the fecal-oral route, ingestion of the infective cyst form occurs via contaminated food and water. In the pre-invasive form of the disease, motile trophozoites, resulting from excystation in the small intestine or colon, interact with the mucin layer that forms the body's first line of defense. In the invasive stage of amoebiasis, *E. histolytica* trophozoites breach the mucus secreting epithelium of the human colon and encounter the submucosa, which is comprised of loose connective tissue, blood vessels and extracellular matrix (ECM) components, including collagen and fibronectin. Destruction of epithelium and the ECM that surrounds the epithelial cells produces flask shaped ulcers [1]. The resultant manifestations, including diarrhea and dysentery, are major

public health concerns in developing and underdeveloped countries. In some cases, colonic invasion can result in dissemination of trophozoites to extra-intestinal sites like liver, lungs and brain through the portal vascular system, resulting in amoebic abscess [1]. Thus, adhesion to ECM components and their subsequent degradation facilitates invasion and is a critical step in the pathogenesis of amoebiasis.

Several lines of evidence suggest that adhesion of *E. histolytica* to ECM may be likened to focal adhesions of higher eukaryotes [2, 3]. This interaction is also believed to alter signal transduction pathways and enhance parasitic virulence [2, 4, 5]. For example, exposure of *E. histolytica* trophozoites to collagen induces actin accumulation and phosphorylation of tyrosine residues on the *E. histolytica* homolog of $pp125^{FAK}$ [3, 6, 7]. $pp125^{FAK}$ is a non receptor cytosolic focal adhesion kinase (FAK) that localizes to adhesion plaques and is activated by tyrosine phosphorylation [6]. Exposure of *E. histolytica* to collagen also stimulates $pp125^{FAK}$ association with paxillin and Src (pp^{60src}), which may result in increased DNA binding of the transcription factor AP-1 [7]. Collagen also induces phosphorylation of $p42^{MAPK}$, which may propagate a phosphorylation-based signal from the plasma membrane to the nucleus [6]. In addition, exposure of trophozoites to collagen has been shown to increase DNA binding of several other *E. histolytica* transcription factors such as STAT1 and STAT3 [4]. This, in turn, may regulate changes in gene expression. In support of this, collagen exposure results in increased expression of an amoebapore and a cysteine protease, two secreted proteins which aid in host tissue destruction [5]. Likewise, binding of *E. histolytica* trophozoites to fibronectin induces phosphorylation of FAK, association of an integrin like receptor

with paxillin, and activation of protein kinase A (PKA) [8, 9], a protein involved in G-protein coupled receptor signaling. Additionally, exposure to fibronectin induces reorganization of actin and its redistribution to the sites of adhesion [2, 10]. Since exposure to ECM components may upregulate signaling events that modulate virulence, a better understanding of adhesion to ECM may provide insight into pathogenic mechanisms.

Recent evidence suggests that there exist highly-ordered cholesterol- and sphingolipid-rich microdomains, termed lipid rafts, in the plasma membrane of *E. histolytica*. These are thought to play an important role in endocytosis, secretion and adhesion of the parasite to host cells [11]. In higher eukaryotes, lipid rafts serve as signaling platforms in which integrins, as well as other adhesion/signaling molecules, may reside or accumulate in a signal-dependent fashion [11-13]. The existence and physiological relevance of these rafts in *E. histolytica* has been shown through a variety of methods, including fluorescence microscopy, using fluorescent lipid analogs which preferentially intercalate into ordered membrane domains, raft disruption using specific cholesterol-binding reagents, and biochemical isolation and characterization of membrane microdomains [11]. That a previous study indicated that the galactose and N-acetyl-D-galactosamine inhibitable lectin (Gal/GalNAc lectin), an adherence lectin of *E. histolytica*, is localized to rafts, supports the role of such domains during adhesion to host components [11]. The Gal/GalNAc lectin is composed of a 170 kDa transmembrane heavy chain subunit (Hgl) linked via a disulphide bond to a light subunit (Lgl; 31-35 kDa); which is GPI-anchored. These subunits are non-covalently associated with a 150 kDa intermediate subunit (Igl)

[reviewed in 14]. The Gal/GalNAc lectin has been postulated to bind to galactose and N-acetylgalactosamine residues of host glycoconjugates on mucin, epithelial cells and erythrocytes [15-17]. In light of these observations, and in light of the fact that ECM components are glycosylated, it is conceivable that lipid raft-resident molecular components, like the Gal/GalNAc lectin, may also be involved in adhesion to host ECM.

The present study provides insight into the involvement of lipid rafts in adhesion of *E. histolytica* trophozoites to elements of the host ECM. Here we demonstrate that disruption of rafts inhibits adhesion to host ECM, and that lipoprotein supplementation enhances adhesion. Using fluorescence microscopy, we show that raft membranes accumulate at the trophozoite-ECM interface. Treatment with galactose also inhibits adhesion to collagen, which may implicate the Gal/GalNAc lectin in this important adhesion event. However, adhesion to fibronectin appears to occur independent of the Gal/GalNAc lectin, though adhesion to fibronectin may be partially mediated by lipid rafts.

III. Materials and Methods

Strains and culture conditions

E. histolytica trophozoites, strain HM-1:IMSS, were cultured axenically in TYI-S-33 medium in screw-cap glass tubes at 37°C [18]. Log phase harvested trophozoites were used for all experiments. Chinese hamster ovary (CHO) cells were cultured at 37°C in 25 cm^2 angle-necked cell culture flasks in Dulbecco's modified Eagle's medium

(Biowhittaker, Walkersville, MD) supplemented with fetal bovine serum (10% v/v), 1M HEPES (Invitrogen, Carlsbad, CA), and penicillin-streptomycin (Invitrogen) (1% v/v).

Measurement of adhesion to CHO cells

CHO cells have been previously used as a model for host epithelium [19, 20]. To test the effect of cholesterol on adhesion to host cells, we used a standard adhesion assay described by Powell *et al*. [21]. CHO cells were grown to confluency in 96-well plates. The CHO monolayer was then fixed with 4% paraformaldehyde to prevent cytolysis, washed twice with PBS, incubated in 250 mM glycine to inactivate residual paraformaldehyde activity and then washed twice with PBS. Log phase *E. histolytica* trophozoites were iced for 8 minutes to dislodge them from glass, pelleted by centrifugation (500 x *g* for 5 minutes) and then resuspended in prewarmed TYI-33 media. Trophozoites were then dispensed in 15 ml conicals and labeled with 5 μg/ml Calcein AM (Invitrogen), a green fluorescent vital stain, at 37°C for 60 minutes. Some of these trophozoites were treated with the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich, St. Louis, MO), during the last 30 minutes of Calcein AM staining. In all cases, MβCD was dissolved in TYI-33 media (TYI-S-33 media without serum) to attain a final concentration of 15 mM. Untreated control cells, as well as raft-disrupted cells were then centrifuged (500 x *g* for 5 minutes) and re-suspended in media with or without bovine lipoprotein-cholesterol concentrate (LCC) (MP Biomedicals, Solon, OH) and incubated at 37°C for 15 minutes. LCC was used at a concentration that provided 0.5 μg/ml cholesterol. 3 X 10^4 control cells as well as treated cells were then seeded onto the CHO monolayer. Following incubation at 37°C for 30 minutes, non

adherent cells were removed by gently washing the wells twice with warm phosphate-buffered saline (PBS). The relative fluorescence, as a measure of adhesivity, was assessed using a fluorescence plate reader (Model FLX800, BioTek Instruments, Winooski, VT). The excitation and emission wavelengths used were 485 nm and 528 nm, respectively.

Measurement of *E. histolytica* adhesion to ECM

A standard assay for measuring adhesion to host epithelial cells was adapted for measuring adhesion to ECM [21]. Log phase trophozoites, labeled with Calcein AM, as described above, were seeded onto commercial collagen type I- or fibronectin-coated 96-well plates (BD Biosciences, Bedford, MA) at increasing concentrations, from 1 X 10^4 to 20 X 10^4 cells per well. Following incubation at 37°C for 15 minutes, unbound cells were washed and the level of adhesion was measured by spectrofluorimetry. Alternatively, the number of adherent cells was determined by counting 5 fields per well by examination at a magnification of 40X on an Olympus CK2 inverted light microscope. The number of cells to be seeded into the wells for subsequent experiments, as well as the incubation time, was determined empirically by examining a range of cell concentrations and a range of incubation times. To test the role of lipid rafts in adhesion to ECM, adhesion assays, as described above were performed with cells that were treated with a range of concentrations of MβCD, and/or 0.5 μg/ml LCC.

Lipid raft staining

Trophozoites were allowed to adhere to glass (control), collagen type I- or fibronectin-coated cover slips (BD Biosciences) in serum-free medium. Following incubation at 37°C for 15 minutes, the medium was aspirated and the non adherent cells were removed by washing twice with warm PBS. The cells were fixed by treatment with 4% (v/v) paraformaldehyde for 10 minutes at room temperature. After fixing, cells were washed twice with PBS, and then incubated with the fluorescent lipid raft stain, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ($DiIC_{16}$) [22] (4.5 mM; Molecular Probes, Eugene, OR), for 10 minutes. The cover slips were then washed twice with PBS, mounted in PBS and observed using a Zeiss LSM 510 confocal microscope.

Galactose-mediated inhibition of amoebic adhesion to ECM

To test if the Gal/GalNAc lectin is involved in adhesion to ECM, Calcein AM stained trophozoites were incubated on collagen type I- or fibronectin-coated plates in the presence of a range of concentrations of D(+) Galactose (Sigma-Aldrich) from 10 mM to 100 mM. Galactose was dissolved in TYI-33 media to obtain the appropriate concentration. As a control, adhesion was also tested in presence of 100 mM Mannose (Sigma-Aldrich) [15, 16], which was also dissolved in TYI-33 media.

Statistical Analyses

All values represent the mean ± standard deviations [SD] of at least three trials. Statistical analyses were performed using GraphPad Instat (version 3.05; IBM) with one-way ANOVA with post-tests. *P*-values less than 0.05 were considered statistically significant

and were denoted by a single asterisk (*). *P*-values less than 0.01 were considered highly statistically significant and were denoted by two (**) asterisks. The mean inhibitory dose (IC_{50}) was calculated using the line of best fit generated by TableCurve2D version 5.01 (Systat).

IV. Results

Development of a high throughput adhesion assay for quantifying adhesion of *E. histolytica* to collagen and fibronectin

In order to assess the role of lipid rafts in parasite-host ECM interaction, we developed a high-throughput assay for quantifying this cellular function. To this end, we adapted a standard adhesion assay used to measure adhesion of *E. histolytica* to host epithelial cells [21]. Trophozoites were stained with Calcein AM, a membrane permeant compound that is metabolized by intracellular esterases in live cells into a membrane impermeant fluorescent cytoplasmic dye [23]. To determine if Calcein AM staining inhibits adhesion to ECM, an equal number of Calcein AM-stained or unstained cells were added to the wells of collagen type I- or fibronectin-coated microtiter plates. The number of adherent cells was determined by counting 5 fields per well using a light microscope. It was observed that there was no significant difference between the number of adherent cells with or without treatment with Calcein AM (Fig. 2.1), suggesting that staining with Calcein AM does not impact adhesivity of cells to ECM.

Calcein AM stained cells were then added, in increasing numbers, to successive wells of the coated plates. After incubation for 15 minutes, non adherent cells were removed by gentle washing. The level of adhesion was quantified by assessing fluorescence intensity

of adherent cells using spectrofluorimetry. As cell number increased from 1 X 10^4 cells per well to 5 X 10^4 cells per well, the relative fluorescence intensity increased linearly. Beyond this concentration, a plateau was observed (Fig. 2.2).

Therefore 5 X 10^4 cells per well represents the maximum number of cells that can adhere likely due to the limited surface area of a single microtiter well. To refine standard adhesion curves, we repeated the adhesion assays using cell concentrations only in the linear range of the initial graph, that is, 1 X 10^4 to 5 X 10^4 cells per well (Fig. 2.3). The statistically significant linear increase in relative fluorescence within this range of cell concentrations suggests that this assay authentically quantifies adhesion of *E. histolytica* to ECM components. From these standard curves, we determined 2.5 X 10^4 cells per well to be a median cell number which was used for all subsequent experiments. Using a median cell number would allow us to observe both decreases and increases in adhesion to ECM. In preliminary experiments, a range of incubation times, from 15 minutes to 2 hours were tested (data not shown). At the 15 minute time point, fluorescence intensity increased maximally with increasing cell number and therefore this time point was used for all further assays.

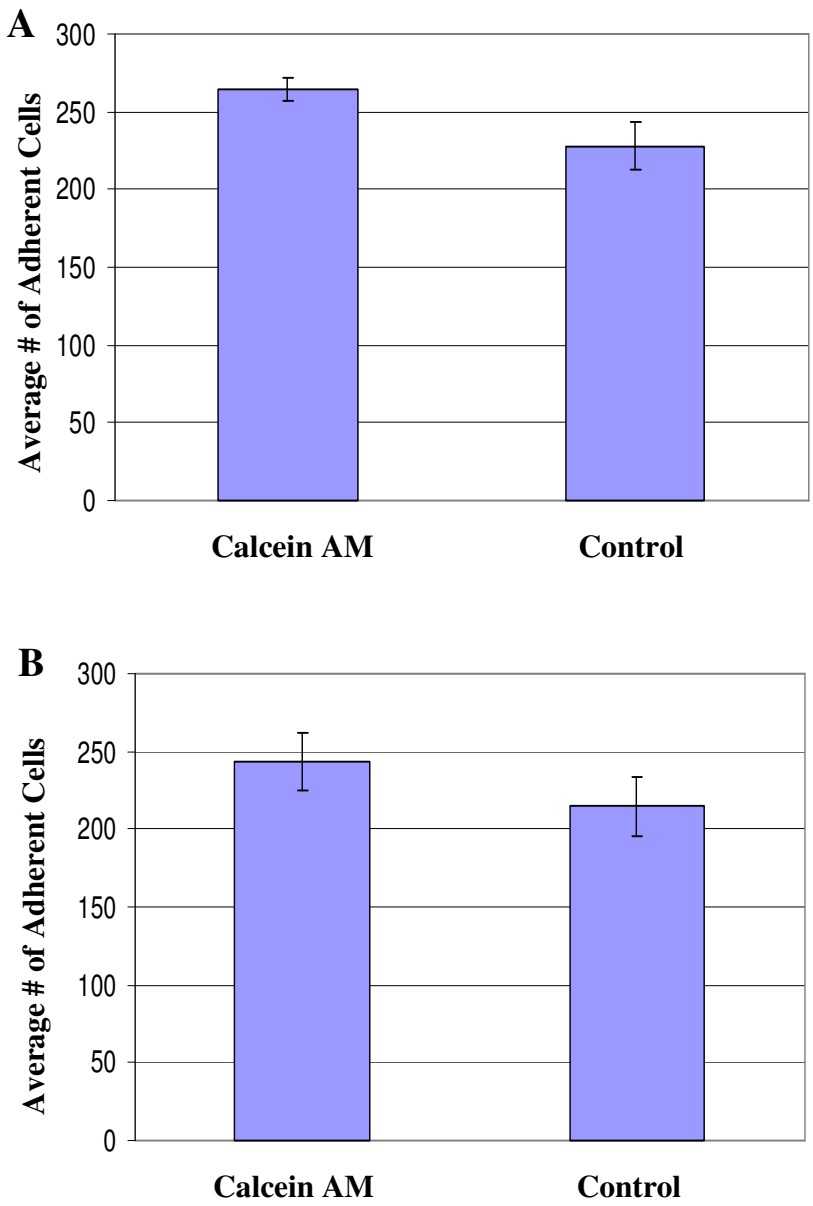

Figure 2.1. Calcein AM does not inhibit adhesion of *E. histolytica* to ECM.
2.5 X 10^4 Calcein AM stained and unstained control cells were incubated in the wells of (A) collagen- and (B) fibronectin-coated 96-well microtiter plates. Adherent cells were counted by naked eye using a light microscope in 5 fields per well in triplicate. The level of adhesion of Calcein AM stained cells was not significantly different from that of unstained control cells. The results represent the mean ± standard deviation of three trials for (A) collagen ($P > 0.065$) as well as (B) fibronectin ($P > 0.5$). Calcein AM does not inhibit adhesion of *E. histolytica* to ECM.

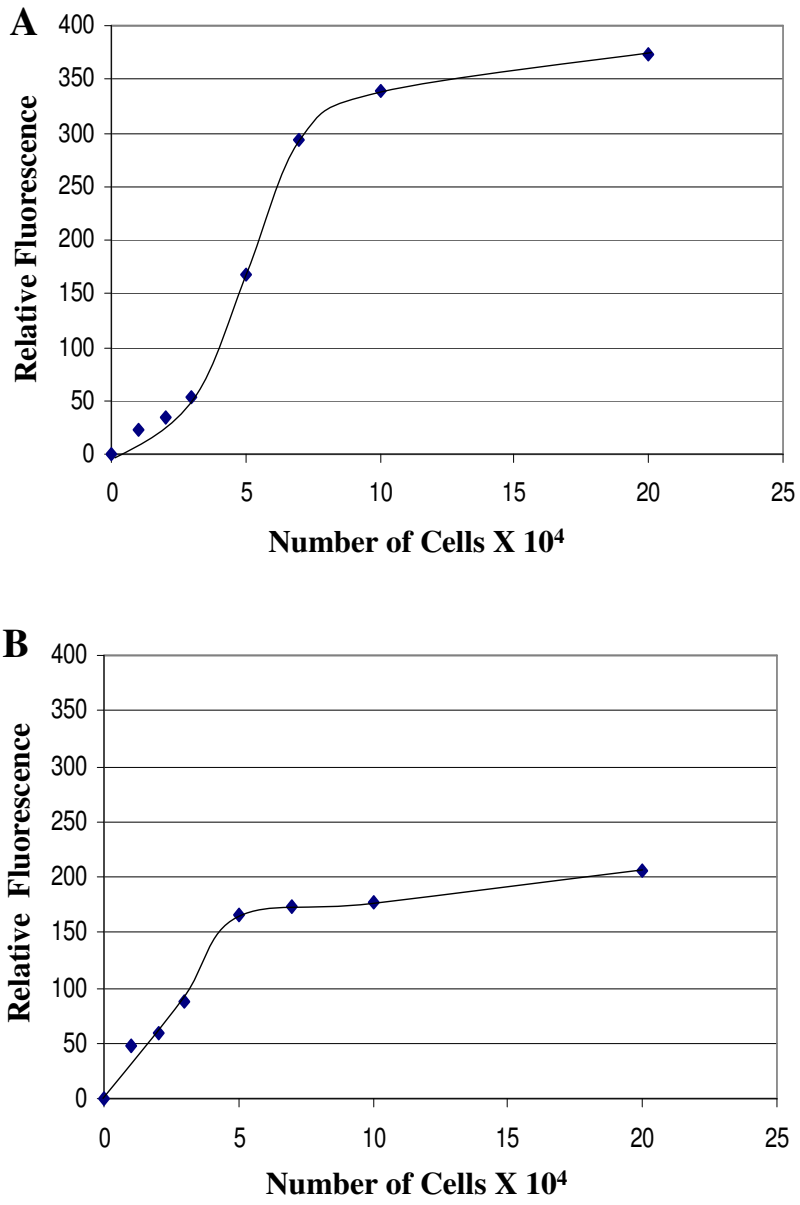

Figure 2.2. Adhesion of *E. histolytica* to ECM.
Calcein AM-stained *E. histolytica* cells were seeded in increasing numbers in the wells of (A) collagen- and (B) fibronectin-coated 96-well microtiter plates. Relative fluorescence increased linearly with increasing cell concentration from 1 X 10^4 to 5 X 10^4 cells per well. Fluorescence intensity did not increase significantly at concentrations greater than 5 X 10^4 cells per well, suggesting that this concentration represents the maximum number of cells that can adhere to a microtiter well surface. The values represent the mean from triplicate wells in a single representative experiment.

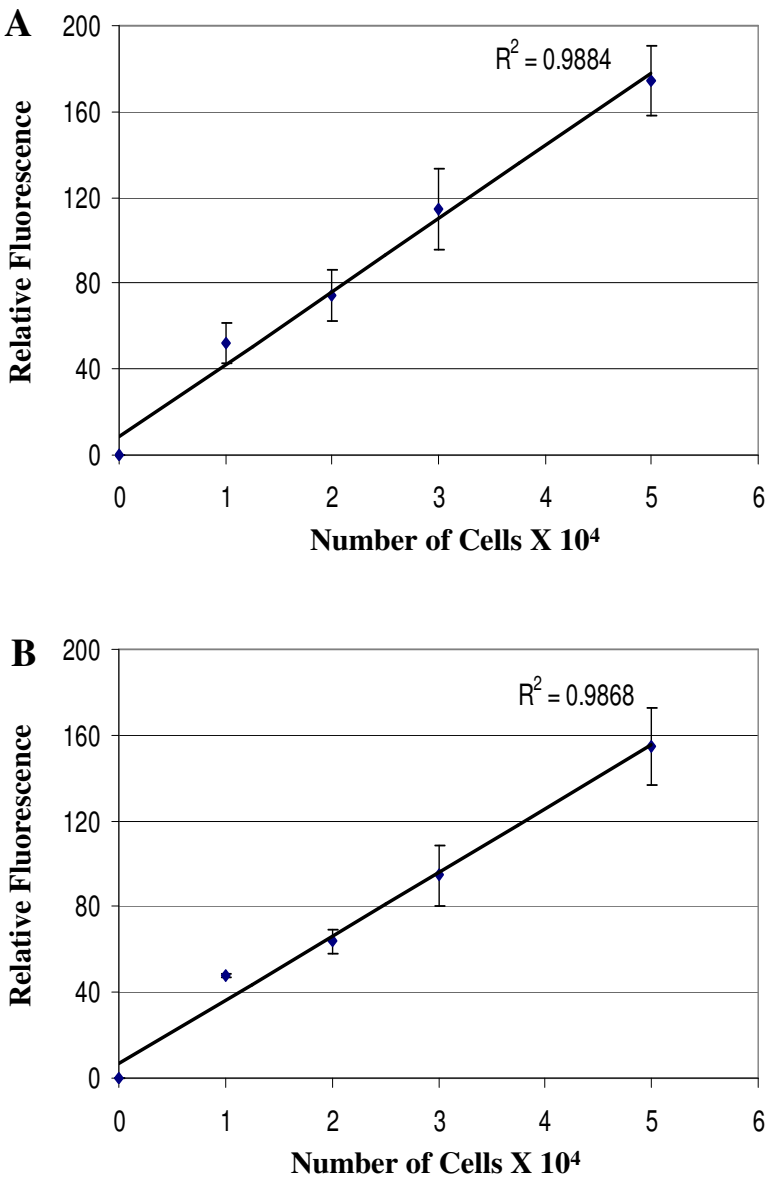

Figure 2.3. Standard adhesion curves for *E. histolytica* adhesion to ECM.
Calcein AM-stained *E. histolytica* cells were seeded in increasing numbers into wells of (A)collagen- and (B)fibronectin-coated 96-well microtiter plates at concentrations from 1 X 10^4 to 5 X 10^4 cells per well. Relative fluorescence was measured by spectrofluorimetry. Fluorescence intensity increases linearly with cell number. The data represent the mean ± standard deviation of 3 trials for (A) collagen (R^2=0.9884) and 4 trials for (B) fibronectin (R^2=0.9868).

Extracellular lipoprotein reverses MβCD-induced inhibition of *E. histolytica* adhesion to host cells

Recent evidence suggests that *E. histolytica* trophozoites bind to host epithelial cells through cholesterol- and sphingolipid-rich membrane microdomains known as lipid rafts. The evidence includes the observation that disruption of these microdomains, using MβCD, inhibits adhesion to host epithelial cells [11]. MβCD is a surface-acting cyclic heptasaccharide that selectively extracts membrane cholesterol by reversibly encapsulating it in a central hydrophobic core [24, 25]. To further explore the role of cholesterol-rich membrane in adhesion to host cells and to explore the specificity of MβCD for future experiments, we tested the effect of cholesterol addition on adhesion of trophozoites to host cells. Since it has been reported that *E. histolytic*a cells cannot use free cholesterol [26], we treated trophozoites with lipoprotein-cholesterol concentrate (LCC) and measured their ability to adhere to host epithelial cells. Adhesion to host cells was significantly increased in the presence of LCC. This further supports the involvement of cholesterol in adhesion to host epithelium and suggests that this concentrate may be useful to test the role of cholesterol-rich membrane in adhesion to other surfaces.

We then treated trophozoites with MβCD alone or MβCD followed by LCC. Consistent with previous results [11], MβCD significantly reduced adhesion to host cells by 47.5% (Fig. 2.4). Interestingly, treatment with LCC restored MβCD-induced adhesion to near normal levels. This observation supports the authenticity of the mode of action of MβCD as a cholesterol-sequestering agent. Therefore, MβCD may also be a useful agent to test the role of cholesterol-rich membrane in adhesion to other surfaces. The concentration of LCC used provided 0.5 μg/ml cholesterol. Since this concentration restored MβCD-

induced inhibition of adhesion to near normal levels, this concentration was used in all subsequent experiments. For both treated and untreated cells, more than 86% of cells remained viable during the experiments, as determined by trypan blue exclusion (data not shown). This suggests that observed changes in adhesion were a physiological effect of cholesterol depletion, and not a result of decreased viability of the cells.

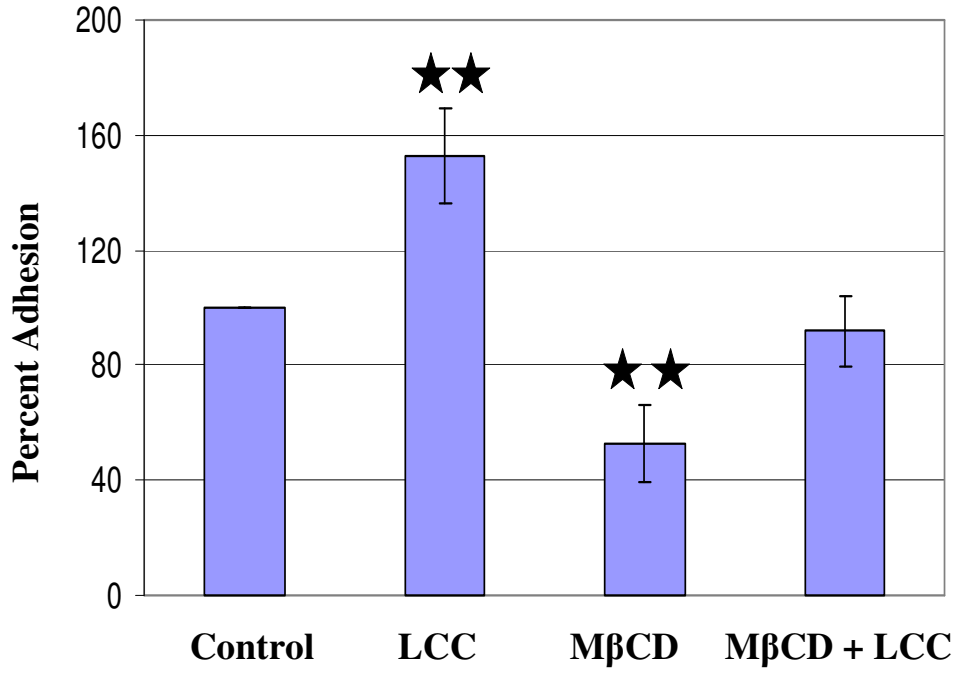

Figure 2.4. Restoration of MβCD-induced inhibition of adhesion on host epithelial cells by LCC treatment.
Adhesion of Calcein AM-stained cells pretreated with lipoprotein-cholesterol (LCC**;** 0.5 µg/ml cholesterol), Methyl-β-cyclodextrin (MβCD**;** 15mM), or MβCD treatment followed by treatment with lipoprotein-cholesterol (MβCD + LCC) was quantified by spectrofluorimetry. The data are presented as a percentage of adhesion of untreated control cells which was arbitrarily set to 100%. The level of adhesion after lipoprotein-cholesterol treatment or after MβCD treatment was significantly different from control (n=3,***P*<0.01). The level of adhesion of cells treated with MβCD followed by LCC was not significantly different from control. Treatment with LCC enhances adhesion to host cells and can reverse MβCD-induced inhibition of adhesion.

MβCD decreases adhesion to ECM in a dose-dependant fashion

To determine if lipid rafts are involved in adhesion to host ECM components, collagen and fibronectin, we assessed adhesion of trophozoites to ECM-coated plates after biochemical disruption of lipid rafts using MβCD as described above and previously [11]. Adhesion to collagen and fibronectin decreased, in a dose-dependant fashion, after treatment with MβCD (Fig. 2.5). The mean inhibitory concentration (IC_{50}) of MβCD for collagen was determined to be 27 mM. It is noteworthy that while, at a concentration of 15 mM, MβCD was able to inhibit adhesion to collagen by 43.66%, it was only able to inhibit adhesion to fibronectin by 27.17%. Although this was a statistically significant decrease ($P<0.05$), lipid rafts play a lesser role in adhesion to fibronectin than to collagen.

Extracellular lipoprotein reverses MβCD-induced inhibition of adhesion to ECM

In order to assess the specific effect of membrane cholesterol level on adhesion to ECM, control cells and cholesterol depleted cells were suspended in media supplemented with or without LCC. Subsequently, their ability to adhere to host ECM was determined using the fluorescence-based assay described above. Treatment with LCC alone increased adhesion to both host substrates (Fig. 2.6, 2.7). MβCD treatment inhibited adhesion to collagen by 60%. While adhesion to fibronectin was decreased by 44% after raft disruption, this decline was not found to be statistically significant. This supports our previous observation suggesting lipid rafts play a lesser role in adhesion to fibronectin, and that raft-independent mechanisms must also participate in this process. Treatment of raft-disrupted cells with LCC restored adhesion to both collagen and fibronectin (Fig 2.6, 2.7). This suggests that cholesterol-rich membrane is important in adhesion to host ECM

and that MβCD-induced inhibition of adhesion is, in fact, the result of loss of lipid. We cannot rule out the possibility that another component of LCC enhanced adhesion may have over-ridden MβCD induced inhibition of adhesion. However, others have used repletion of cholesterol after raft disruption as a successful approach to demonstrate the role of lipid rafts in various physiological processes [27-29]. Overall, our observations support the notion that cholesterol-rich membrane may be involved in adhesion to host ECM.

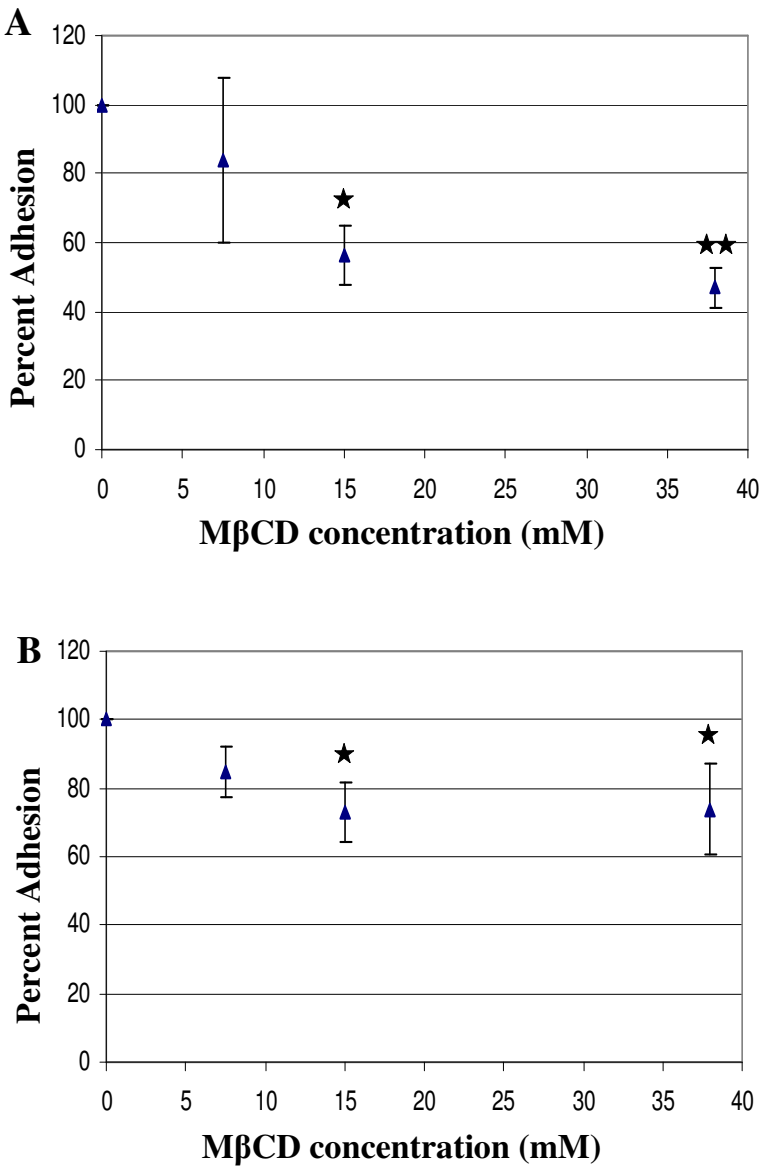

Figure 2.5. MβCD-mediated inhibition of adhesion to collagen and fibronectin.
Calcein AM-treated *E. histolytica* trophozoites were treated with a range of concentrations of MβCD prior to incubation on (A) collagen and (B) fibronectin surfaces. The data are presented as a percentage of adhesion of cells not treated with MβCD (0 mM) which was arbitrarily set to 100%. MßCD inhibits adhesion of trophozoites to both ECM components in a dose-dependant fashion (n=3). *P*-values less than 0.05 are considered statistically significant and are denoted by a single asterisk (*). *P*-values less than 0.01 are considered highly statistically significant and are denoted by two (**) asterisks.

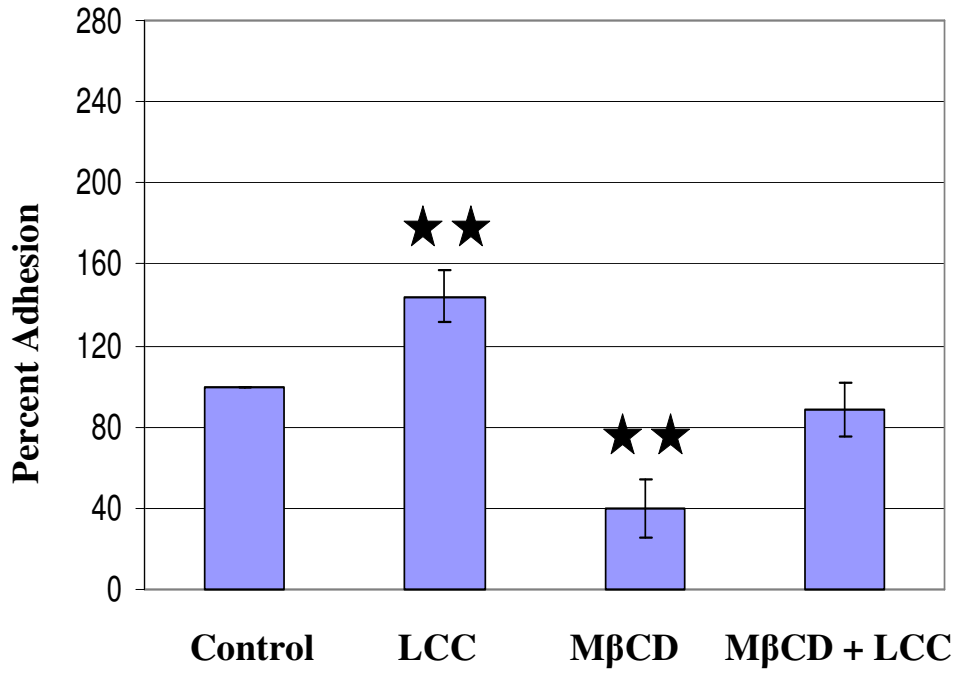

Figure 2.6. Raft disruption and exposure to lipoprotein affect adhesion to collagen. Adhesion of Calcein AM-stained cells pretreated with lipoprotein-cholesterol concentrate (LCC**;** 0.5 μg/ml cholesterol), methyl-β-cyclodextrin (MβCD**;** 15mM), or MβCD treatment followed by treatment with LCC (MβCD + LCC) was quantified by spectrofluorimetry. The data are presented as percentage of adhesion of untreated control cells which was arbitrarily set to 100%. The level of adhesion after LCC treatment or after MβCD treatment was significantly different from control (n=3, $^{**}P<0.01$). The level of adhesion for cells treated with MβCD followed by LCC was not significantly different from control. Treatment with LCC can reverse MβCD-induced inhibition of adhesion to collagen.

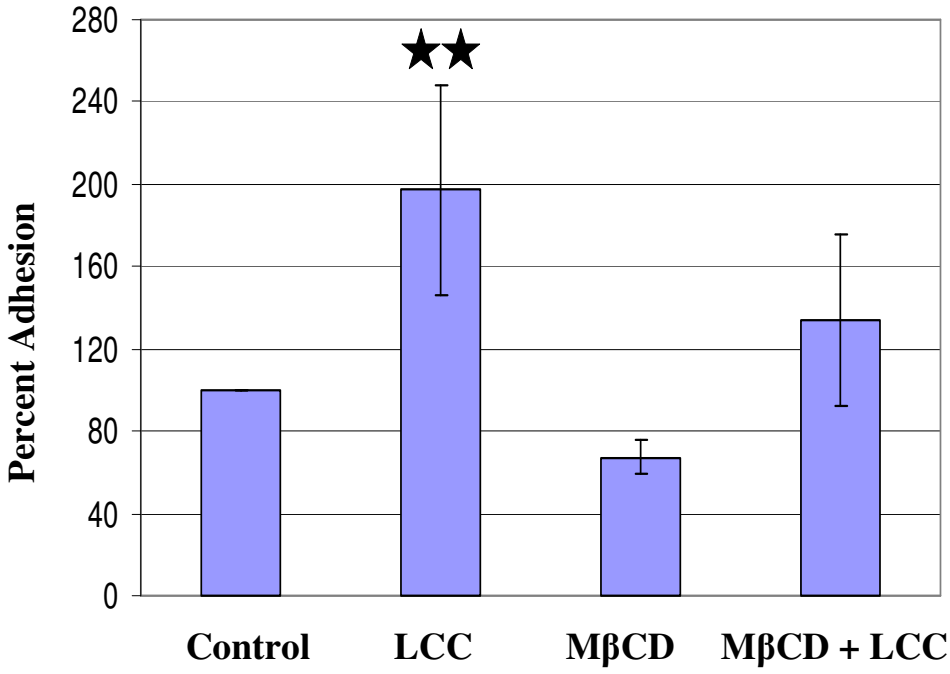

Figure 2.7. Raft disruption and exposure to lipoprotein affect adhesion to fibronectin. Adhesion of Calcein AM-stained cells pretreated with lipoprotein-cholesterol concentrate (LCC**;** 0.5 μg/ml cholesterol), methyl-β-cyclodextrin (MβCD**;** 15mM), or MβCD treatment followed by treatment with LCC (MβCD + LCC) was quantified by spectrofluorimetry. The data are presented as percentage of adhesion of untreated control cells which was arbitrarily set to 100%.The level of adhesion after treatment with LCC was significantly different from control (n=4, $^{**}P<0.01$). The level of adhesion after treatment with MβCD or MβCD + LCC was not significantly different from that of control (n=4, $P>0.05$). Treatment with LCC can reverse MβCD-induced inhibition of adhesion to fibronectin.

Raft microdomains accumulate at the site of parasite–ECM contact

Since raft-disrupting agents inhibit adhesion, and since lipoprotein enhances adhesion to ECM, it is conceivable that rafts might accumulate at the parasite-ECM interface. To determine if raft microdomains enrich at these contact sites, we allowed *E. histolytica* trophozoites to adhere to ECM coated cover slips and stained these cells with the fluorescent lipid raft stain, $DiIC_{16}$ [22]. Raft microdomains were found to accumulate at the parasite-ECM interface, thereby supporting a role for these microdomains in parasite-ECM interactions (Fig. 2.8, 2.9). In contrast, $DiIC_{16}$-stained domains were not particularly enriched at the contact site of trophozoites with glass. Rather, in these control cells, $DiIC_{16}$-stained domains were observed to be distributed uniformly throughout the plasma membrane of the cell (Fig. 2.10). This supports the authenticity of our microscopic observations of trophozoites on ECM surfaces.

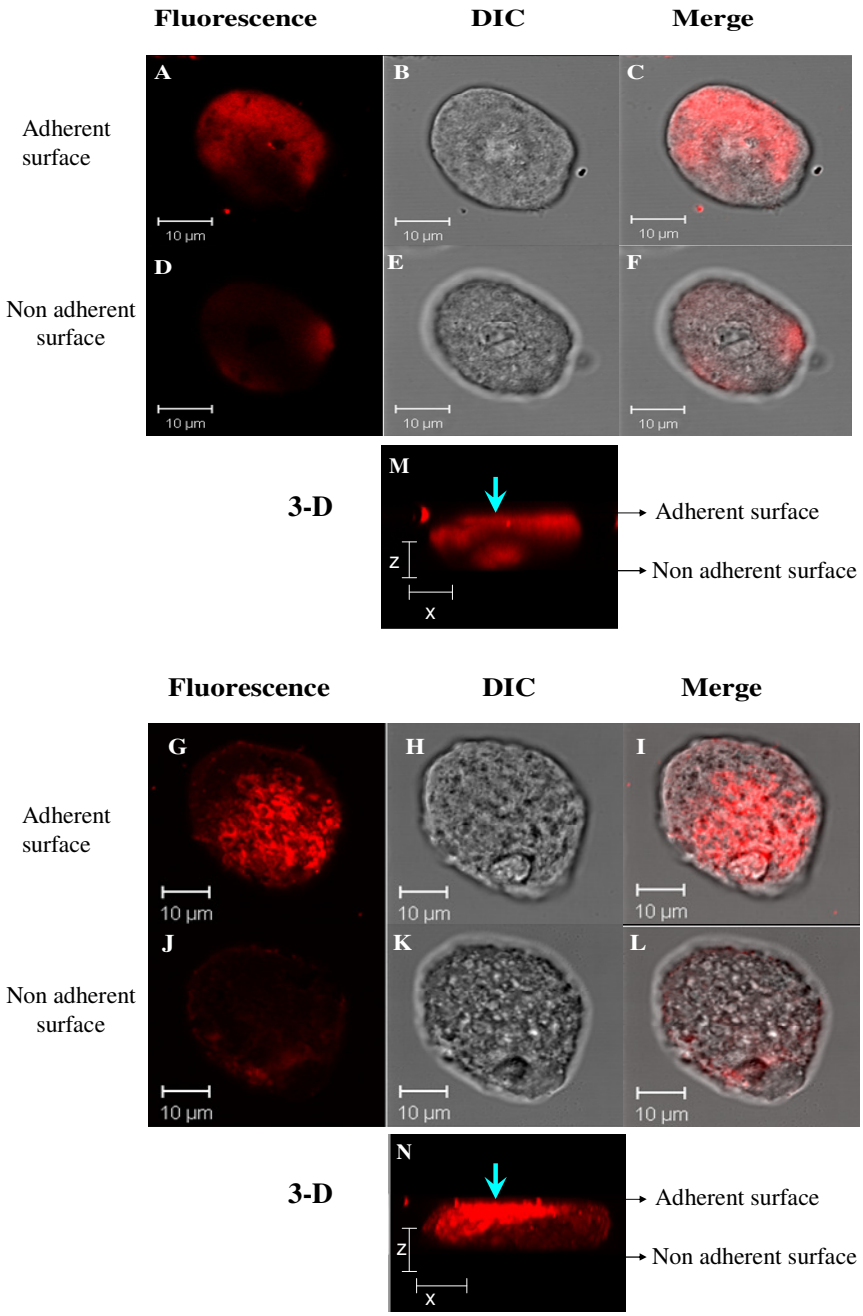

Figure 2.8. Lipid raft enrichment at parasite-collagen interface.
Fluorescence microscopy images of $DiIC_{16}$-stained cells adhering to collagen demonstrate the adherent surface of 2 cells (A, G) in x-y plane, enriched in lipid rafts. Panels D, J represent the non adherent surface of the cell. M, N are 3 dimensional reconstructions viewed in x-z plane indicating the accumulation of lipids at the interface (depicted by arrow). B, E, H and K represent differential interference contrast (DIC) images while C, F, I and L represent the merged images respectively. Scale bars represent 10 µm.

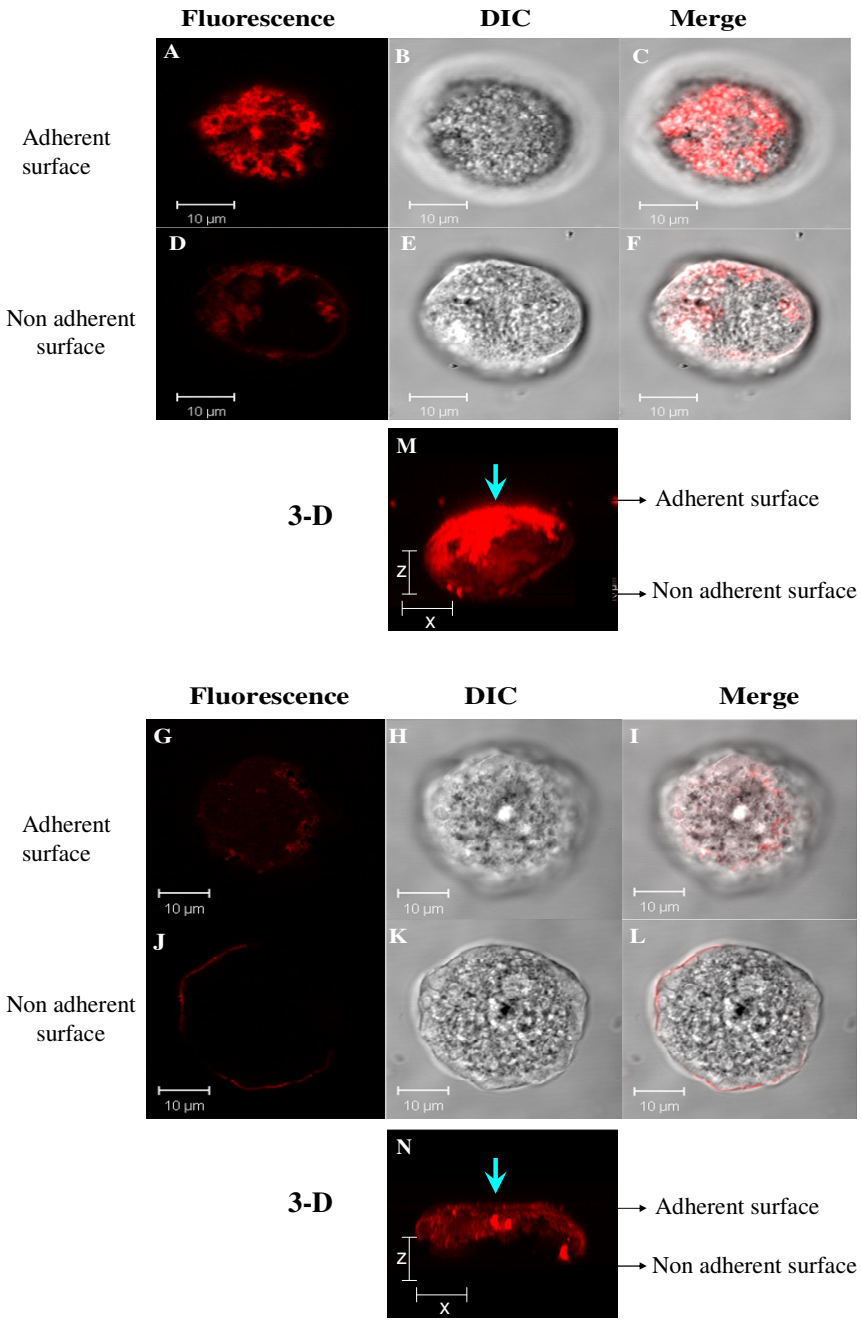

Figure 2.9. Lipid raft enrichment at parasite-fibronectin interface.
Fluorescence microscopy images of $DiIC_{16}$ stained cells adhering to fibronectin demonstrate the adherent surface of 2 cells (A, G), in x-y plane, enriched in lipid rafts. Panels D, J represent the non adherent surface of the cell. M, N are 3 dimensional reconstructions viewed in x-z plane indicating the accumulation of lipids at the interface (depicted by arrow). B, E, H and K represent differential interference contrast (DIC) images while C, F, I and L represent the merged images respectively. Scale bars represent 10 µm.

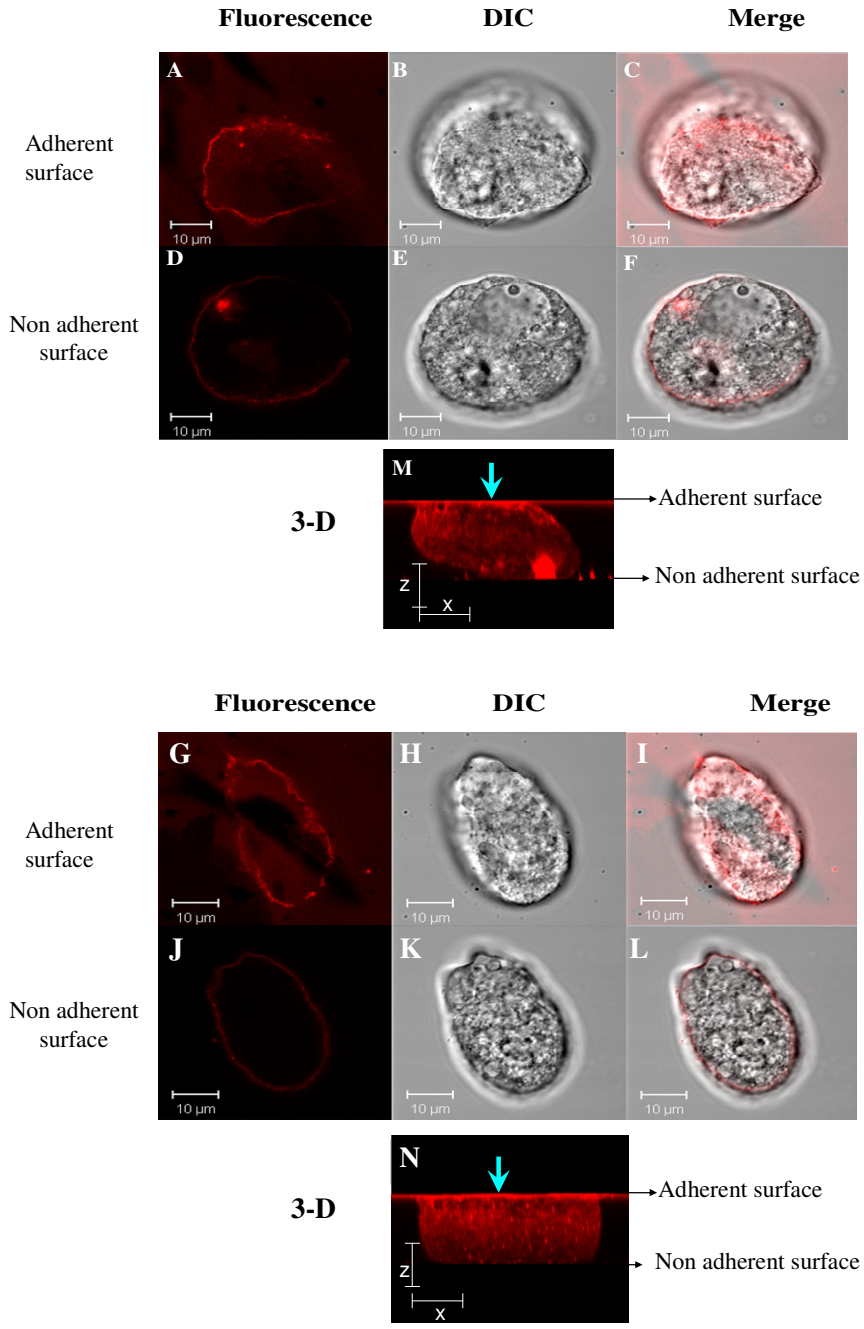

Figure 2.10. Lipid rafts do not enrich at the interface of glass.
Fluorescence microscopy images of $DiIC_{16}$ stained cells adhering to glass demonstrate the adherent surface of 2 cells (A, G), in x-y plane. Panels D, J represent the non adherent surface of the cell. M, N are 3 dimensional reconstructions viewed in x-z plane indicating the presence of $DiIC_{16}$-staining domains throughout the cell membrane. Arrow depicts parasite-ECM interface. B, E, H and K represent differential interference contrast (DIC) images while C, F, I and L represent the merged images respectively. Scale bars represent 10 µm.

The Gal/GalNAc lectin is involved in adhesion to collagen, but not to fibronectin

Since lipid rafts are involved in adhesion of the parasite to ECM, it is conceivable that the receptors for collagen and fibronectin reside within lipid rafts. To date, only one receptor has been demonstrated to reside in rafts, that is, the Gal/GalNAc lectin [11]. It has been previously demonstrated that the Gal/GalNAc lectin is involved in adhesion of *E. histolytica* to host glycoconjugates [15, 16]. To determine if the Gal/GalNAc lectin regulates adhesion to collagen and fibronectin, we quantified trophozoite adhesion to ECM-coated plates in the presence of a range of concentrations of galactose. As a control, adhesion was tested in the presence of 100 mM mannose, which has been shown to exert a non inhibitory effect on adhesion to host cell glycoconjugates [15, 16]. Our results indicated that galactose significantly decreases adhesion to collagen in a dose- dependant fashion, while mannose exerts no significant effect on adhesion (Fig. 2.11 A). This suggests that the Gal/GalNAc lectin may be a putative receptor within the lipid rafts that is involved in adhesion of *E. histolytica* to collagen. The mean inhibitory concentration (IC_{50}) for collagen was determined to be 30 mM. However, galactose was found to exert no significant inhibitory effect on adhesion of trophozoites to fibronectin (Fig. 2.11 B), suggesting that receptors other than the Gal/GalNAc lectin may be involved in adhesion to this substrate. This receptor or receptors may reside in raft or non-raft regions of the cell.

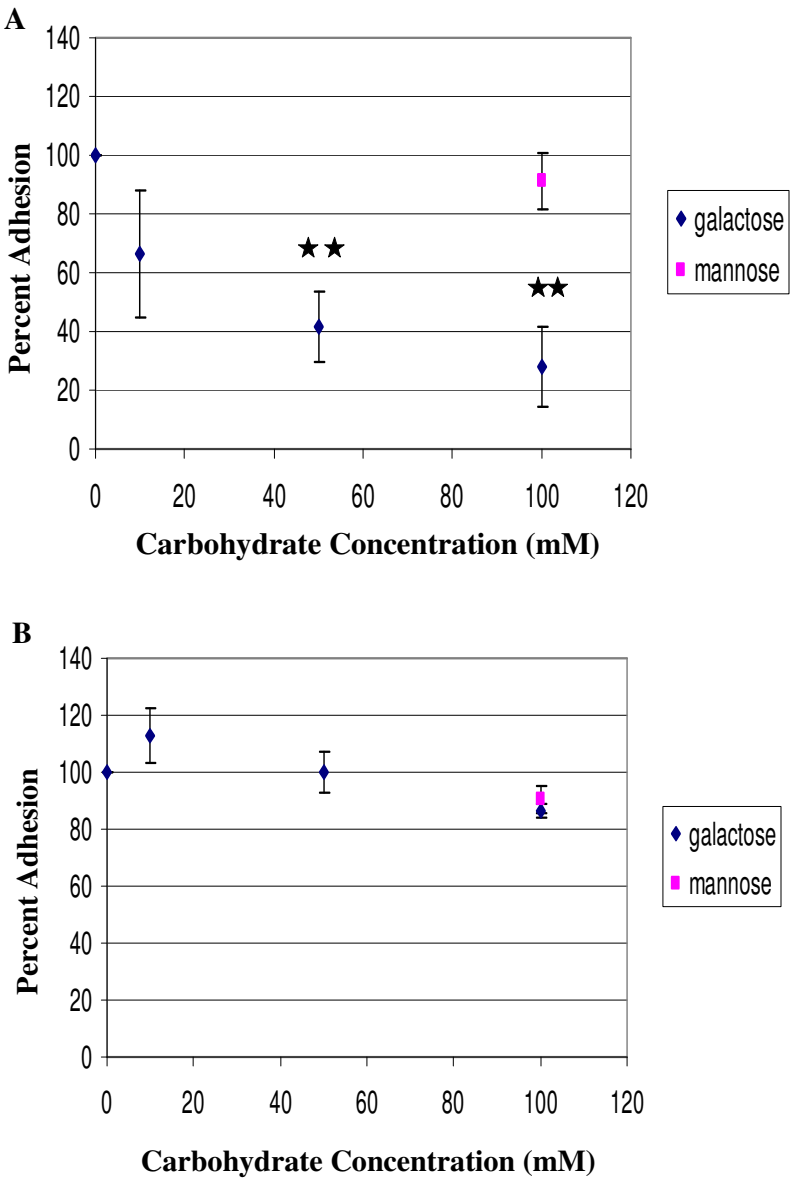

Figure 2.11. Galactose inhibits adhesion to collagen but not to fibronectin.
Calcein AM treated cells were incubated onto (A) collagen- and (B) fibronectin-coated 96 well plates in the presence of a range of concentrations of galactose (10-100 mM) or 100 mM mannose. After washing non adherent cells, adhesion was quantified by spectrofluorimetry. The data are expressed as a percentage of adhesion of untreated control cells which was arbitrarily set to 100%. The data represent mean ± standard deviation of 3 trials for collagen and 4 trials for fibronectin. Galactose inhibits adhesion to collagen in a dose dependant fashion. Galactose does not significantly inhibit adhesion to fibronectin. Mannose exerts no significant effect on adhesion to either ECM component ($P > 0.05$). *P*-values less than 0.01 are considered highly statistically significant and were denoted by two (**) asterisks.

V. Discussion

A key step in the pathogenesis of invasive amoebiasis is incursion of *E. histolytica* into the lamina propria, which brings trophozoites in contact with extracellular matrix (ECM) [30]. Since exposure to ECM components may upregulate signaling events as described previously, an understanding of the molecular mechanisms of adhesion to ECM may provide insight into disease pathogenesis. In this study, we have investigated the role of lipid rafts in the interaction of *E. histolytica* with ECM elements, collagen and fibronectin. We observed a dose-dependant decrease in adhesion of trophozoites to collagen and a lesser decrease in adhesion to fibronectin, as a consequence of raft disruption. Our results indicated that supplementation with cholesterol by treatment with lipoprotein-cholesterol concentrate (LCC) leads to enhanced adhesion to ECM. LCC treatment also rescues the decline in adhesion observed for raft disrupted cells. Together, these data suggest that cholesterol-rich membrane participates in adhesion to collagen, and to a lesser extent, fibronectin. In support of this, using fluorescence microscopy, we have observed the enrichment of lipid rafts at the parasite-ECM interface. Finally, the Gal/GalNAc lectin, a resident of lipid rafts, may be a putative receptor for adhesion to collagen, but is unlikely to be involved in adhesion to fibronectin.

Membrane rafts are defined as highly dynamic sterol- and sphingolipid-enriched domains that compartmentalize cellular processes [31]. The physiological role of lipid rafts in cellular adhesion has been the subject of numerous studies, including a study in *E. histolytica* [11]. Previous research also suggests a positive correlation between the membrane cholesterol levels and adhesivity of cells. In fibroblasts transformed with

polyoma virus (PyF), an increase in membrane cholesterol content was shown to restore the transformation related loss of adhesivity [32]. Huang *et al*. [33] recently demonstrated that disruption of lipid rafts in human cancer cell lines inhibits cellular adhesion to fibronectin, collagen and laminin, thereby affecting a crucial step in tumor invasion and metastasis. In a murine system, detergent resistant fractions were found to be associated with brain-derived tenascin glycoproteins of the ECM [34], supporting the notion that the interaction of cells with ECM components might be raft-mediated. We present similar evidence suggesting, for the first time, a role of lipid rafts in adhesion of *E. histolytica* to host collagen and to a lesser extent, fibronectin.

Epidemiological data has demonstrated that patients with non invasive amoebiasis (cyst passers) have lower cholesterol levels in comparison to patients with invasive amoebiasis (amoebic liver abscess) [35]. A region around Hue´ City in central Vietnam has reported an extraordinarily high number of male patients with amoebic liver abscess. [36]. It is remarkable that according to the WHO Global Infobase [37], the mean serum cholesterol level in males in Vietnam (5.4 mM/L total cholesterol) is higher than that in males in other areas where amoebiasis is endemic, like India (5.1 mM/L) and Mexico (4.8 mM/L). It is not our intention to oversimplify the contribution of cholesterol to the outcome of amoebiasis, and we realize that confounding factors such as differences in nutrition, general poor health, and other co-morbid conditions may also contribute to the pathogenesis of disease. However, it is still interesting that the most common site for manifestation of extraintestinal amoebiasis is the liver, which is also the primary site for cholesterol synthesis in the human body [38]. Laboratory evidence indicates that

lipoprotein enrichment can support *E. histolytica* growth in serum free media [39]. Since trophozoites are unable to utilize free cholesterol *in vitro* [26], it is possible that *in vivo*, cholesterol-enriched lipoprotein particles in the colonic lumen, tissue and/or serum act as a source of cholesterol in successive stages of invasive disease [38]. In support of this notion, it has been reported that a gradual decline in the ability of trophozoites to induce hepatic abscess in hamsters occurs on prolonged growth in culture. More importantly, this decrease in virulence is reversed on passage through hamster liver or through growth in cholesterol-rich media [40]. Also, unpublished data from our laboratory indicate that treatment with lipoprotein-cholesterol may enhance other virulence functions of *E. histolytica* like erythrophagocytosis and host cell cytolysis in a dose dependant manner. Therefore, host cholesterol levels might regulate parasitic virulence, but the exact mechanisms by which host lipids might influence pathogenecity must still be investigated. Indeed, enhanced adhesion, as a result of cholesterol exposure, as shown in this study, may be a contributory factor.

Diabetes has also been postulated to be a risk factor for amoebiasis [41]. In a retrospective study conducted in Taiwan, patients with diabetes mellitus were found to have a greater incidence of severe amoebic liver abscess [42]. Likewise, amongst patients of amoebiasis in Mexico, a frequent disease association between incidence of diabetes and the development of fulminant amoebic colitis was established [43]. The latter is a rare complication of amoebiasis, carrying high morbidity and mortality [44]. The role of diabetes as a prognostic factor in amoebiasis has been explained by the compromised immunity in diabetic patients, and also due to the microangiopathy that occurs in the

intestinal vasculature in diabetics [41]. Given the well established association between hyperglycemia and hypercholesterolemia, we hypothesize that high cholesterol levels may be an additional mechanism which may contribute to a worse disease outcome in diabetic patients with amoebiasis.

Curiously, raft disruption is unable to abolish adhesion completely. For instance, at a dose of 15 mM MβCD, there was only a 44% observed decline in adhesion of trophozoites to fibronectin. An explanation for this might lie in the proposed hypothetical model for organization of sphingolipids, cholesterol and GPI-anchored proteins based on their behavior after treatment with MβCD [45]. According to this model, a small fraction of cholesterol, in the core of sphingolipid-rich domains, is resistant to extraction by MβCD. Pucadyil *et al.* [46] investigated the membrane cholesterol content of macrophages after treatment with 10 mM MβCD and found only a 40% decline in cholesterol at this treatment concentration. Thus, it is possible that lipid rafts are not completely impaired by MβCD treatment, which could account for the residual adhesion. On the other hand, incomplete inhibition of adhesion may suggest that raft independent mechanisms may also be involved in adhesion to ECM.

Despite a lesser decline in adhesion of raft-disrupted trophozoites to fibronectin than to collagen, it is noteworthy that LCC prominently enhances adhesion to this ECM component. Thus, there exists a possibility that at least one receptor for adhesion to fibronectin may not rely on rafts, but lipid rafts still play a partial role in interaction with fibronectin. An interesting observation has been the restoration of adhesion to near-

normal levels in raft disrupted cells upon LCC treatment. This supports the notion that the defect in adhesion in raft-disrupted cells is due to a reversible loss of lipids. Again, we cannot disregard the possibility that LCC enhances adhesion by a mechanism exclusive of lipid rafts, and the reversion of cellular adhesion is merely an additive outcome of two independent mechanisms.

To visualize the cellular interface of the parasite with ECM and to gain a better understanding of cell-ECM interactions, a variety of approaches have been utilized in the past. One of these strategies included the use of three-dimensional ECM matrices that enabled observation of cells such as neutrophils [47] and T lymphocytes [48] interacting with ECM. In *E. histolytica*, adhesion to collagen and fibronectin is believed to simulate formation of focal adhesions similar to those in higher eukaryotes [2, 3]. Recently, a novel strategy, employing the use of atomic force microscopy, has revealed formation of adhesion plaques when *E. histolytica* trophozoites adhere to fibronectin-coated cover slips [49]. Previously, fluorescent lipid raft stain, $DiIC_{16,}$ was used to demonstrate the existence of raft domains in the plasma membrane of *E. histolytica* [11], and we utilized a similar approach to study the parasite's adhesion to ECM. We observed an enrichment of lipid raft stain at the parasite-ECM interface on collagen- or fibronectin-coated cover slips using fluorescence microscopy. In contrast, cells adherent to glass did not exhibit accumulation of rafts at the site of adhesion, and $DiIC_{16}$-stained domains appeared to be distributed throughout the cell membrane in these control cells. We believe that these findings reflect the specific involvement of lipid rafts in interaction with ECM. Since adhesion, invasion and subsequent degradation of ECM is the natural sequence of events

in the pathogenesis of amoebiasis, it is conceivable that trophozoites would adhere and subsequently attempt to embed themselves in the thin ECM layer on the pre-coated cover slip. For each of the surfaces, the optical parameters were adjusted to ensure that staining at the non adherent surface of the cell was absent. Thus, a quantitative comparison of $DiIC_{16}$-stained domains at the interface with collagen and fibronectin cannot be made. A suggested improvement over this technique would be to maintain uniform laser parameters while imaging cells adherent to different ECM surfaces. It would also be interesting to observe migration patterns of *E. histolytica* through 3-D ECM matrices *in vitro*, since this might allow for quantification of ECM degradation.

The molecular components within the raft that may be involved in adhesion are still under investigation. The most commonly implicated protein in adhesion mechanisms is the Gal/GalNAc lectin. It has been established that the Gal/GalNAc lectin binds to galactose and N-acetylgalactosamine residues on host glycoconjugates and thus mediates adherence and cytotoxicity of the parasite [15, 16, 50, 51]. The heavy subunit of this lectin, the Hgl, contains a carbohydrate recognition domain [14] and monoclonal antibodies against this subunit of lectin inhibit adhesion to host cells [52]. An important discovery has been that of enrichment of the Gal/GalNAc lectin heavy subunit in the detergent resistant membrane fraction, that is, the lipid raft [11]. In this study, we observed a dose-dependant, galactose-mediated, inhibition of adhesion to collagen. A control sugar, mannose did not have a significant effect on adhesion. This finding suggests the involvement of Gal/GalNAc lectin, perhaps as a raft-resident protein, in

adhesion to collagen. Since galactose did not significantly inhibit adhesion to fibronectin, interaction with fibronectin does not involve the Gal/GalNAc lectin.

Numerous studies have indicated that integrins are responsible, in part, for mammalian cell-ECM interactions [53]. Previous reports demonstrate the existence of an integrin-like molecule in *E. histolytica* that mediates interactions with fibronectin as well as collagen [54]. It is proposed that a 140 kDa β1 integrin-like molecule (*Eh*FNR), upon adhesion to fibronectin, assembles a multimolecular complex that activates signaling pathways within the cell [8]. Interestingly, it has also been demonstrated recently that the *Eh*FNR bears greater than 96% sequence homology with the C terminal domain of the intermediate chain of the Gal/GalNAc lectin (Igl) [54]. Another subunit of the Gal/GalNAc lectin, the Hgl also exhibits homology to β2 and β7 mammalian integrins in its C terminus [20]. The exact relationship between integrins and lipid raft, and more specifically, the *Eh*FNR, the Gal/GalNAc lectin and lipid raft is still unclear. Yet, this study has offered evidence implicating lipid rafts in adhesion to both ECM components, and has substantiated the involvement of Gal/GalNAc lectin in adhesion to collagen. Future studies may generate a more comprehensive picture investigating hitherto unknown receptors, both within, and outside the rafts, that interact with ECM and aid in invasion of the host.

VI. Literature cited

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