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### Entamoeba histolytica:

Lipid rafts are involved in adhesion of trophozoites to host extracellular matrix components

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#### **Abstract**

Adhesion is an important virulence function for *Entamoeba histolytica*, the causative agent of amoebic dysentery. Lipid rafts, cholesterol-rich domains, function in compartmentalization of cellular processes. In *E. histolytica*, rafts participate in parasite-host cell interactions; however, their role in parasite-host extracellular matrix (ECM) interactions has not been explored. Disruption of rafts with a cholesterol extracting agent, methyl- $\beta$ -cyclodextrin (M $\beta$ CD), resulted in inhibition of adhesion to collagen, and to a lesser extent, to fibronectin. Replenishment of cholesterol in M $\beta$ CD-treated cells, using a lipoprotein-cholesterol concentrate, restored adhesion to collagen. Confocal microscopy revealed enrichment of rafts at parasite-ECM interfaces. A raft-resident adhesin, the galactose/N-acetylgalactosamine inhibitible lectin, mediates interaction to host cells by binding to galactose or N-acetylgalactosamine moieties on host glycoproteins. In this study, galactose inhibited adhesion to collagen, but not to fibronectin. Together these data suggest that rafts participate in *E. histolytica*-ECM interactions and that raft-associated Gal/GalNAc lectin may serve as a collagen receptor.

#### Keywords

adhesion; cholesterol; collagen; Entamoeba histolytica; extracellular matrix; fibronectin

#### 1. INTRODUCTION

Entamoeba histolytica is a human intestinal pathogen that affects more than 50 million people worldwide, leading to 100,000 deaths annually. (reviewed in Laughlin and Temesvari, 2005). 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. 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 ECM components, including collagen and fibronectin. Destruction of epithelium and the ECM that surrounds the epithelial cells produces flask shaped ulcers [Laughlin and Temesvari, 2005). 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 such as the liver,

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lungs, and brain through the portal vascular system (Laughlin and Temesvari, 2005). 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 [Talamas-Rohana and Meza, 1988; de Lourdes et al., 2001). This interaction is also believed to activate parasite signal transduction pathways and virulence (Talamas-Rohana and Meza, 1988; de Lourdes et al., 2001; Cruz-Vera et al., 2003; Debnath et al., 2004; Perez et al., 1996; Perez et al., 1998; Flores-Robles et al., 2003; Franco et al., 2002; Talamas-Rohana and Rios, 2000). For example, exposure of E. histolytica to collagen induces both actin reorganization within the trophozoites and phosphorylation of tyrosine residues on the E. histolytica homolog of pp125FAK (de Lourdes et al., 2001; Perez et al., 1996). pp125<sup>FAK</sup> is a cytosolic FAK that localizes to adhesion plaques (Perez et al., 1996). Exposure of E. histolytica to collagen also stimulates pp125<sup>FAK</sup> association with paxillin and Src (Perez et al., 1998) and phosphorylation of p42MAPK (Perez et al., 1996), a map kinase which may propagate a collagen-based signal from the plasma membrane to the nucleus. Encountering collagen also increases DNA binding of three E. histolytica transcription factors, AP-1 (Perez et al., 1998), STAT1, and STAT3 (Cruz-Vera et al., 2003), which, 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 (Debnath et al., 2004), two secreted proteins which play a role in host tissue destruction. Binding of E. histolytica trophozoites to a second ECM component, fibronectin, also induces actin reorganization (Talamas-Rohana and Meza, 1988; Talamas-Rohana and Rios, 2000), phosphorylation of pp125<sup>FAK</sup> (Flores-Robles et al., 2003), pp125<sup>FAK</sup> association with paxillin and vinculin (Flores-Robles et al., 2003), and activation of protein kinase A (Franco et al., 2002), an enzyme involved in G-protein coupled receptor signaling. Since exposure to ECM components appears to upregulate signaling events that modulate virulence, a better understanding of adhesion to ECM may provide insight into pathogenic mechanisms.

Recent evidence suggests that highly-ordered cholesterol- and sphingolipid-rich microdomains, termed lipid rafts, are present in the plasma membrane of E. histolytica (Laughlin et al., 2004). In higher eukaryotes, rafts are thought to harbor specialized transmembrane, GPI-linked, and dually acylated proteins (reviewed in Maxfield, 2002; Simons and Toomre, 2000). They are also thought to serve as signaling platforms in which adhesion/ signaling molecules, such as integrins, accumulate in a signal-dependent fashion (Maxfield, 2002; Simons and Toomre, 2000). In E. histolytica, rafts have been shown to play a role in endocytosis and adhesion to host cells (Laughlin et al., 2004). In addition, an important adherence protein of E. histolytica, the Gal/GalNAc lectin, is localized to these membrane microdomains (Laughlin et al., 2004). The Gal/GalNAc lectin is a heterotrimer comprised of light, intermediate, and heavy subunits, and binds to galactose and N-acetylgalactosamine residues of host glycoconjugates on mucin, epithelial cells, and erythrocytes (Adler et al., 1995; Raydin and Guerrant, 1981; Petri et al., 2002). Given that rafts participate in cell-matrix interactions in other systems (Ramprasad et al., 2007; Gopaladrishan et al., 2000), it is conceivable that lipid rafts may also play a role in the interaction of E. histolytica with host ECM. Moreover, lipid raft-resident adhesion molecules, such as the Gal/GalNAc lectin, may mediate this function of rafts. In the present study, we have examined the role of lipid rafts in adhesion of E. histolytica trophozoites to elements of host ECM. Here, we demonstrate that disruption of rafts inhibits adhesion to host ECM, and that rafts accumulate at parasite-ECM interfaces. Moreover, we demonstrate that the Gal/GalNAc lectin may mediate interactions to collagen but not to fibronectin. Together these data suggest a role for rafts and their resident proteins in E. histolytica interaction with host ECM.

#### 2. MATERIALS AND METHODS

#### 2.1 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 (Diamond et al., 1978). Log phase harvested trophozoites were used for all experiments.

#### 2.2 Measurement of E. histolytica adhesion

To assess parasite-ECM interactions, we developed a fluorescence-adapted assay based on a similar test used to assess the binding of *E. histolytica* trophozoites to host epithelial cells (Powell et al., 2006). *E. histolytica* trophozoites were labeled with 5 μg/ml calcein AM (Invitrogen, Carlsbad, CA), a green fluorescent vital stain, at 37°C, for 60 min in serum-free medium (TYI-33). These cells were then seeded onto commercial collagen type I- or fibronectin-coated 96-well plates (BD Cellware, Bedford, MA). Following incubation at 37°C, the wells were gently washed twice with warm PBS to remove non-adherent cells. The relative fluorescence (a measure of adhesivity) was assessed using a fluorescence plate reader (Model FLX800, BioTek Instruments, Winooski, VT). Alternatively, the number of adherent cells was determined by counting 5 fields per well at a magnification of 400X 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, adhesion assays were performed with trophozoites that had been treated with a range of concentrations of the cholesterol depleting agent, M $\beta$ CD (Sigma-Aldrich, St. Louis, MO), during the last 30 min of calcein AM staining. Alternatively, adhesion assays were performed with untreated or M $\beta$ CD-treated cells that were exposed to a cholesterol source, lipoprotein-cholesterol concentrate (LCC) (57 mg of cholesterol/dl) (MP Biomedicals, Solon, OH), for 15 min at 37°C. Analysis by the vendor of the lot used throughout the study indicated that LCC consisted of cholesterol (1548 mg/dL), triglycerides (7%), protein (4.5 mg/dL), chloride (50 milliequivalent units/L), ammonia (56  $\mu$ M), calcium (5 mg/dL), and sodium (98 milliequivalent units/L). If applicable, adhesion data were corrected for slight losses in fluorescence due to M $\beta$ CD treatment.

To test if the Gal/GalNAc lectin was involved in adhesion to ECM, adhesion assays were performed in the presence of a range of concentrations of D(+) galactose (Sigma-Aldrich) or 100 mM mannose (control) (Sigma-Aldrich) (Adler et al., 1995; Ravdin and Guerrant, 1981). In all cases, adhesion data were reported as a percent of control, which was arbitrarily set to 100%.

#### 2.3 Confocal Microscopy

Trophozoites were allowed to adhere to collagen type I- or fibronectin-coated cover slips (BD Biosciences) in serum-free medium. Following incubation at 37°C for 15 min, 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% (vol/vol) paraformaldehyde for 10 min at room temperature and then incubated with the fluorescent lipid raft stain, DiIC<sub>16</sub> (Nguyen and Hildreth, 2000) (4.5 mM; Invitrogen), for 10 min. The cover slips were then washed twice with PBS, mounted in PBS, and observed using a LSM 510 confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Adhesion of trophozoites to uncoated glass cover slips was imaged as a control.

#### 2.4 Statistical Analyses

All values are represented as the mean of the results from at least three trials ( $\pm$  standard deviations [S.D.]). Statistical analyses were performed using GraphPad Instat (version 3.05; IBM) with ANOVA. *P*-values less than 0.05 were considered statistically significant (\*). *P*-values less than 0.01 were considered highly statistically significant (\*\*). The IC<sub>50</sub> values were calculated using the line of best fit generated by Table Curve2D version 5.01 (Systat Software Inc., San Jose, CA).

#### 3. RESULTS

#### 3.1 Development of a high throughput adhesion assay for quantifying adhesion of $\underline{E}$ . <u>histolytica</u> to collagen and fibronectin

To assess the role of lipid rafts in parasite-host ECM interaction, we developed a high-throughput assay for quantifying this cellular function. In particular, we adapted a standard adhesion assay that was used to measure adhesion of *E. histolytica* cells to host epithelial cells (Powell et al., 2006). 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 (Boyle, 2004). To determine if calcein AM staining inhibited 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. The number of unstained adherent cells was not different from calcein AM-stained adherent cells (Fig. 1), suggesting that calcein AM does not affect adhesion to collagen or fibronectin.

Increasing numbers of calcein AM-stained cells were then added to successive wells in the coated plates. After incubation for 15 min, non-adherent cells were removed by gentle washing. The level of adhesion was quantified by assessing fluorescence intensity of adherent cells using spectrofluorometry. The relative fluorescence intensity increased linearly with cell number over a range of concentrations (Fig. 2). This linear increase, which was observed for both ECM-coated surfaces, suggests that this assay authentically assesses adhesion of E. histolytica to ECM components. Based on these observations, a median cell number of  $2.5 \times 10^4$  cells per well was chosen for all subsequent experiments. Incubation times of 15 min were shown to be optimal and were used for all adhesion assays (data not shown).

#### 3.2 A raft-disrupting agent, MBCD, decreases adhesion to ECM in a dose-dependent fashion

MβCD is a surface-acting, cyclic heptasaccharide that selectively extracts membrane cholesterol by encapsulating it in a central hydrophobic core (Irie et al., 1992; Pitha et al., 1988). It has been used in numerous systems, including E. histolytica, to assess the role of raft membrane in cellular processes (Laughlin et al., 2004; Ramprasad et al., 2007; Huang et al., 2006a). To determine if lipid rafts are involved in adhesion of E. histolytica to collagen or fibronectin, we examined adhesion of trophozoites to ECM-coated plates after biochemical disruption of lipid rafts using MβCD as described previously (Laughlin et al., 2004). Adhesion to collagen and fibronectin decreased in a dose-dependent fashion after treatment with MβCD (Fig. 3). For both MβCD-treated and untreated cells, more than 86% of the cells remained viable during the experiments, as determined by trypan blue exclusion (data not shown). This suggests that the observed changes in adhesion were a physiological effect of cholesterol depletion, and not the result of decreased cell viability.

The  $IC_{50}$  of M $\beta$ CD for adhesion of trophozoites to collagen was determined to be 15 mM. However, this concentration of M $\beta$ CD was only able to inhibit adhesion to fibronectin by approximately 82% indicating that adhesion to collagen is more sensitive to raft disruption than adhesion to fibronectin. Together with the observation that cells remained viable during

treatment, these differential sensitivities support the notion that inhibition of adhesion to collagen by M $\beta$ CD is authentic and not the result of nonspecific deleterious effects on cellular processes.

## 3.3 An extracellular source of cholesterol reverses $M\beta$ CD-induced inhibition of adhesion to collagen

To determine whether the M $\beta$ CD-induced inhibition of adhesion to ECM was permanent or reversible, and to determine whether the effects of M $\beta$ CD were due to cholesterol depletion, untreated and raft-disrupted (M $\beta$ CD-treated) cells were exposed to an extracellular source of cholesterol as a means to replenish cellular membranes with cholesterol. Since *E. histolytica* trophozoites cannot use free cholesterol (Mata-Cardenas et al., 2000), and are likely to encounter host cholesterol as a lipoprotein conjugate, we used a commercially available lipoprotein-cholesterol concentrate, LCC, to supply the lipid.

Treatment of control cells with LCC significantly enhanced adhesion to collagen while treatment of raft-disrupted cells with LCC restored adhesion to collagen (Fig. 4A). These data suggest that the inhibitory effects of M $\beta$ CD treatment on parasite-collagen interactions may, indeed, be the result of loss of lipid. The effects of LCC treatment of trophozoites interacting with fibronectin were more variable. Although changes were observed, these were not statistically significant, supporting the finding that the interaction of amoebae with this ECM component is less sensitive to changes in cholesterol.

We cannot rule out the possibility that another component of LCC enhanced adhesion of trophozoites to collagen via a raft-independent mechanism, and that the observed rescue of inhibition of adhesion on collagen was an additive outcome of raft-dependent and raft-independent mechanisms. However, the differential outcome of LCC treatment on adhesion to collagen versus fibronectin supports the authenticity of our observations. Importantly, others have used repletion of cholesterol after raft disruption as a successful approach to demonstrate the specific role of lipid rafts in various physiological processes (Huang et al., 2006a; Zeng and Terada, 2001; Li et al., 2006; Oin et al., 2006).

#### 3.4 Raft microdomains accumulate at the site of parasite - ECM contact

Since raft-disrupting agents inhibited adhesion, it was conceivable that rafts might mediate parasite-ECM interactions, thus accumulating at such sites. To determine if raft microdomains were enriched at these trophozoite-ECM contact sites, we allowed E. histolytica trophozoites to adhere to uncoated or ECM coated cover slips and stained these cells with the fluorescent lipid raft stain, DiIC<sub>16</sub> (Laughlin et al., 2004; Nguyen and Hildreth, 2000). Fluorescence was prominent, and formed a lace-like pattern, at cell surfaces that were in contact with collagen (Fig. 5A,B). In contrast, DiIC<sub>16</sub>-staining was minimally enriched at cell surfaces in contact with fibronectin or uncoated cover slips (Fig. 5C-F). In addition, staining was nominal at the non-adherent surfaces of the cells (Fig. 5G-L). To further explore the accumulation of raft membrane near the ECM contact site, we generated three-dimensional reconstructions using successive planar confocal images and viewed the cells in the X-Z plane. These subsequent images further demonstrated a dramatic accumulation of raft-like membrane on cell membranes near collagen surfaces (Fig. 5M). Moreover, these images revealed a slight accumulation of raft-like membrane on cells near fibronectin surfaces (Fig. 5N). This accumulation was not readily seen in individual focal planes (Fig. 5C,D). Finally, no specific accumulation of raft-like membrane was seen for cells on uncoated surfaces (Fig. 5E,F,K,L,O). Rather, in these control cells, DiIC<sub>16</sub>-stained domains were observed to be distributed uniformly throughout the plasma membrane of the cells. Together, these observations suggest that rafts accumulate at parasite-collagen interfaces, and, to lesser extent, at parasite-fibronectin interfaces. For each of the surfaces, the optical parameters were adjusted to check for any

possible staining at the non-adherent surface. Thus, a quantitative comparison of  $\mathrm{DiIC}_{16}$ stained domains at the interface with collagen and fibronectin cannot be made. However, we believe that our observations reflect the specific involvement of lipid rafts in interaction of E. histolytica cells with collagen.

#### 3.5 The Gal/GalNAc lectin may be involved in adhesion to collagen, but not to fibronectin

Since lipid rafts may be involved in adhesion of the parasite to ECM, it is conceivable that the receptors for collagen and fibronectin reside within these domains. To date, only one receptor, the Gal/GalNAc lectin, has been shown to reside in rafts (Laughlin et al., 2004). Since both collagen and fibronectin are post-translationally galactosylated (Myllyharju, 2005; Uysal et al., 1997) it is also conceivable that the Gal/GalNAc lectin may mediate the interaction of the parasite with these host components. To determine if the Gal/GalNAc lectin regulated adhesion to collagen and fibronectin, we quantified trophozoite adhesion to ECM-coated plates in the presence of a range of concentrations of galactose which would act as a competitive inhibitor of Gal/GalNAc binding. As a control, adhesion was also tested in the presence of 100 mM mannose, which does not inhibit the interactions of *E. histolytica* to host cell glycoconjugates (Adler et al., 1995; Ravdin and Guerrant, 1981).

Our results demonstrated that galactose significantly decreased adhesion to collagen in a dose-dependent fashion, while mannose exerted no significant effect on adhesion to this substrate (Fig. 6A). This suggests that the Gal/GalNAc lectin may be a putative raft-associated receptor for collagen. The  $IC_{50}$  of galactose for this adhesion process was determined to be 30 mM. Galactose was found to exert a minimal inhibitory effect on adhesion of trophozoites to fibronectin (Fig. 6B), suggesting that receptors other than the Gal/GalNAc lectin may be involved in adhesion to this substrate.

#### 4. DISCUSSION

In this study, we have investigated the role of lipid rafts in the interaction of E. histolytica with host ECM elements, collagen and fibronectin. Biochemical disruption of rafts resulted in a dose-dependent decrease in trophozoite adhesion to collagen, and a slight decrease in trophozoite adhesion to fibronectin. LCC treatment of cholesterol-depleted cells also resulted in a nearly complete reversal of the M $\beta$ CD-induced reduction in adhesion to collagen. Together, these data suggest that cholesterol-rich membrane domains participate in adhesion to collagen, and to a lesser extent, fibronectin. In further support of this, fluorescence microscopy demonstrated a significant enrichment of lipid rafts at parasite-collagen interfaces and a lesser enrichment of these microdomains at parasite-fibronectin interfaces. Finally, the Gal/GalNAc lectin, a resident protein of lipid rafts (Laughlin et al., 2004), may mediate adhesion to collagen, but may not be involved in adhesion to fibronectin.

Curiously, biochemical raft disruption was unable to completely abolish adhesion of E. histolytica cells to ECM. For instance, at a dose of 15 mM M $\beta$ CD, there was a  $\sim$ 50% and a  $\sim$ 18% decline in adhesion of trophozoites to collagen or fibronectin, respectively (Fig. 3). An explanation for this might lie in the proposed model for the organization of lipids within rafts (Ilangumaran and Hoessli, 1998). According to this model, a small fraction of cholesterol, in the core of sphingolipid-rich domains, is resistant to extraction by M $\beta$ CD. Pucadyil et al. (2004) investigated the membrane cholesterol content of macrophages after treatment with 10 mM M $\beta$ CD and found only a 40% decline in cholesterol. Thus, it is possible that, in the present study, the cholesterol in the lipid rafts was not completely depleted by M $\beta$ CD treatment, and this may account for the residual adhesion.

On the other hand, incomplete inhibition of adhesion may also suggest that additional raftindependent mechanisms are involved in adhesion to ECM. Given the importance of adhesion

to *E. histolytic*a virulence, it would not be surprising if the pathogen possessed multiple mechanisms of interacting with the host. Various mechanisms may be differentially employed depending on the host component, as well as the state of the host component in question. For example, galactose can block phagocytic uptake of healthy host cells by approximately 80% (Huston et al., 2003), suggesting that the Gal/GalNAc lectin significantly contributes to this process. In contrast, galactose blocks phagocytic uptake of apoptotic host cells by 40% (Huston et al., 2003), indicating that mechanisms other than Gal/GalNac-mediated adhesion participate in parasite-host interactions. Here we show that trophozoites are differentially affected by M $\beta$ CD, LCC and galactose when interacting with collagen or fibronectin suggesting that different mechanisms might regulate these processes.

The differential behavior of trophozoites on collagen and fibronectin, after treatment with M $\beta$ CD or LCC, or in the presence of galactose, also indicates that our observations are not due to nonspecific activity of the reagents. In a previous study, it was shown that 15 mM M $\beta$ CD blocked *E. histolytica* adhesion to host epithelial cells by approximately 25% (Laughlin et al., 2004). In the current study, 15 mM M $\beta$ CD blocked adhesion to collagen by approximately 50%. Therefore, the molecular mechanisms that regulate parasite adhesion to host cells may also be different from those that regulate adhesion to ECM. A complete understanding of *E. histolytica*-host interaction will require insight into all of these modes of interaction.

The role of lipid rafts in cell-ECM interactions has been investigated in a number of other organisms. Importantly, all of these studies demonstrate a positive correlation between the membrane cholesterol levels and adhesivity to ECM substrates. For example, in fibroblasts transformed with polyoma virus, an increase in membrane cholesterol content restores the transformation-related loss of adhesivity (Kaur et al., 2004). In addition, Huang et al. (2006b) demonstrated that disruption of lipid rafts in human cancer cell lines inhibits their adhesion to fibronectin, collagen, and laminin, thereby affecting a crucial step in tumor invasion and metastasis. In a murine system, lipid raft membrane fractions were found to be associated with brain-derived tenascin glycoproteins of the ECM (Kappler et al., 2002). We present similar evidence suggesting, for the first time, a role for lipid rafts in adhesion of *E. histolytica* to host collagen, and to a lesser extent, fibronectin. Therefore, the role of rafts in cell-ECM interactions may be evolutionarily conserved.

In mammalian cells, it is well-established that integrins participate in cell-ECM interactions (Auer and Jacobson, 1995). It has also been reported that many integrins function in the context of lipid rafts (Leitinger and Hogg, 2002; Decker and ffrench-Constant, 2004). For example, the T-cell integrins, LFA-1 and  $\alpha 4\beta 1$ , are excluded from rafts in the absence of stimulation, but move to raft compartments upon activation (Leitinger and Hogg, 2002). In addition, a truncated form of LFA-1, which mimics activated integrin, is constitutively localized to rafts (Leitinger and Hogg, 2002). A  $\beta 1$ -containing integrin of oligodendrocytes (Decker and ffrench-Constant, 2004) also demonstrates raft-enrichment after activation. The clustering of these receptors in rafts is thought to amplify outside-in signaling events and to facilitate the formation of the mammalian focal adhesion (Decker and ffrench-Constant, 2004).

Integrin-like proteins and focal adhesion-like structures also exist in *E. histolytica*. For example, atomic force microscopy has revealed formation of focal adhesion-like plaques when *E. histolytica* trophozoites adhere to fibronectin-coated cover slips (Prazeres et al., 2003). Moreover, a β1 integrin-like molecule, *Eh*FNR, mediates trophozoite interactions with fibronectin and collagen (Hernandez-Ramirez et al., 2007) and assembles a multimolecular complex that includes FAK, paxillin, and vinculin. (Flores-Robles et al., 2003). Hgl, the heavy subunit of the Gal/GalNAc lectin, exhibits homology to β2 and β7 mammalian integrins in its C-terminus (Vines et al., 1998). Indeed, many of *E. histolytica* homologs of mammalian focal adhesion proteins, including actin, pp125<sup>FAK</sup>, paxillin and vinculin, are activated upon

exposure to ECM components (de Lourdes et al., 2001; Perez et al., 1996; Perez et al., 1998; Flores-Robles et al., 2003; Franco et al., 2002). The results of the present study demonstrate that rafts may cooperate with or form a platform for focal adhesion-like structures utilized during interaction with collagen.

Since trophozoites are unable to utilize free cholesterol *in vitro* (Mata-Cardenas et al., 2000), 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 (Bansal et al., 2005). Therefore, it is intriguing to consider the possibility that extracellular lipid exposure might alter pathogenic functions such as adhesion to ECM. Such alterations may influence the success of invasion and amoebic liver abscess formation (ALA). Epidemiological data demonstrating that levels of total serum cholesterol, LDL, and HDL were significantly lower in cyst passers as compared to ALA cases (Bansal et al., 2005) support the importance of cholesterol in invasive disease. Interestingly, in *Giardia*, cholesterol starvation is necessary and sufficient to induce encystation (Slavin et al., 2002). Perhaps, in addition to influencing the success of ALA formation, host cholesterol may also affect processes earlier in infection that commit the parasite to non-invasive or invasive modes of infection.

Although the role of cholesterol-rich rafts in the cellular processes of higher eukaryotic cells is well-established, lipid rafts are only beginning to be recognized as important players of lower eukaryotic cell function. Rafts have been shown to play a role in adhesion of the non-pathogenic amoeba, *Dictyostelium discoideum* (Harris et al., 2001a; Harris et al., 2001b). In the yeast, *Saccharomyces cerevisiae*, polarization of sterol-rich domains was observed in pheromone-induced cells (Bagnat and Simons, 2002). Membrane sterols are also enriched at the leading edge of forming hyphae in the fungal pathogen, *Candida albicans* (Martin and Konopka, 2004). Although the exact relationship between *E. histolytica Eh*FNR, the Gal/GalNAc lectin, and lipid rafts is still unclear, this study offers evidence implicating lipid rafts and the Gal/GalNAc lectin in adhesion of this pathogen to collagen. Future proteomic studies of *E. histolytica* raft and non-raft membrane may generate a more comprehensive picture of receptors, both within and outside the rafts, which interact with ECM and aid in invasion of the host.

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#### **Abbreviations**

DIC, differential interference contrast; DiIC $_{16}$ , 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanineperchlorate; ECM, extracellular matrix; FAK, focal adhesion kinase; Gal/GalNAc lectin, galactose/N-acetylgalactosamine-inhibitable lectin; IC $_{50}$ , mean inhibitory dose; LCC, lipoprotein-cholesterol concentrate; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; PBS, phosphate buffered saline; S.D., standard deviation.

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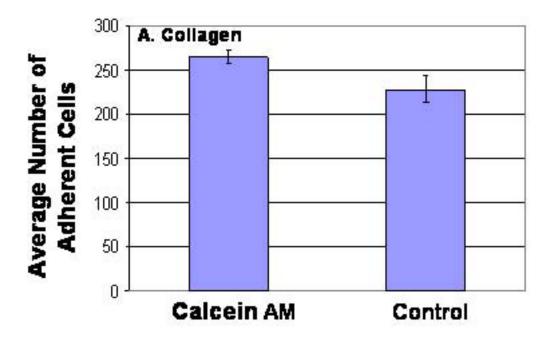
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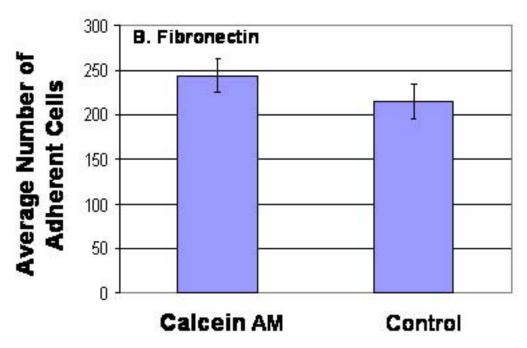


Figure 1. Calcein AM does not inhibit adhesion of *E. histolytica* to ECM Calcein AM stained and unstained control cells  $(2.5 \times 10^4)$  were incubated in the wells of (A) collagen- or (B) fibronectin-coated 96-well microtiter plates. Adherent cells were counted using a light microscope in 5 fields per well in triplicate wells. The level of adhesion of calcein AM stained cells was not significantly different from that of unstained control cells. Therefore, calcein AM does not inhibit adhesion of *E. histolytica* to collagen or fibronectin. The results

represent the mean  $\pm$  S.D. of 3 trials for collagen and fibronectin.

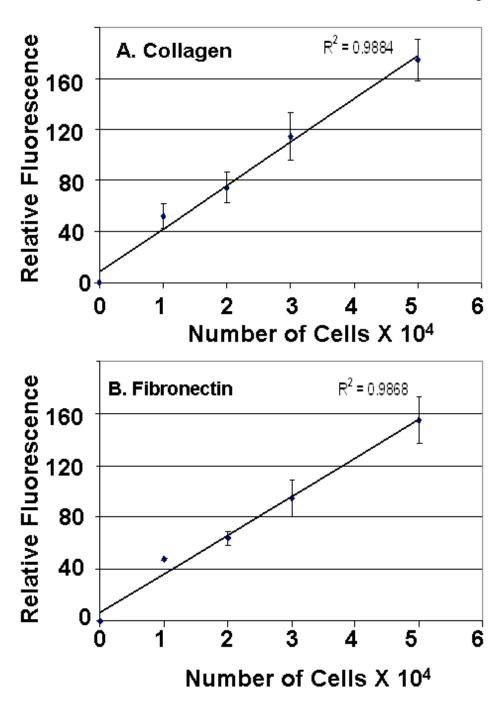


Figure 2. Standard curves for *E. histolytica* adhesion to ECM Calcein AM-stained *E. histolytica* cells were seeded in increasing numbers  $(1 \times 10^4 \text{ to } 5 \times 10^4 \text{ cells per well})$  into wells of (A) collagen- or (B) fibronectin-coated 96-well microtiter plates. Relative fluorescence was measured by spectrofluorimetry and represents a measure of adhesivity. Fluorescence intensity increased linearly with cell number. The data represent the mean  $\pm$  S.D. of 3 trials for collagen (R<sup>2</sup>=0.9884) and 4 trials for fibronectin (R<sup>2</sup>=0.9868).

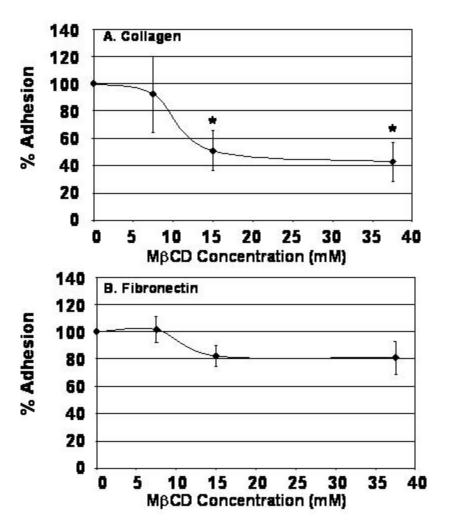


Figure 3. M $\beta$ CD-mediated inhibition of adhesion to collagen and fibronectin Calcein AM-treated *E. histolytica* trophozoites were treated with a range of concentrations of M $\beta$ CD prior to incubation on (A) collagen and (B) fibronectin surfaces. Adhesion was assessed as described in the text. The data are presented as a percentage of adhesion of cells not treated with M $\beta$ CD which was arbitrarily set to 100%. M $\beta$ CD significantly inhibited adhesion of trophozoites to collagen in a dose-dependent fashion (\*P<0.05). The data represent the mean  $\pm$  S.D. of 3 trials for collagen and 4 trials for fibronectin.

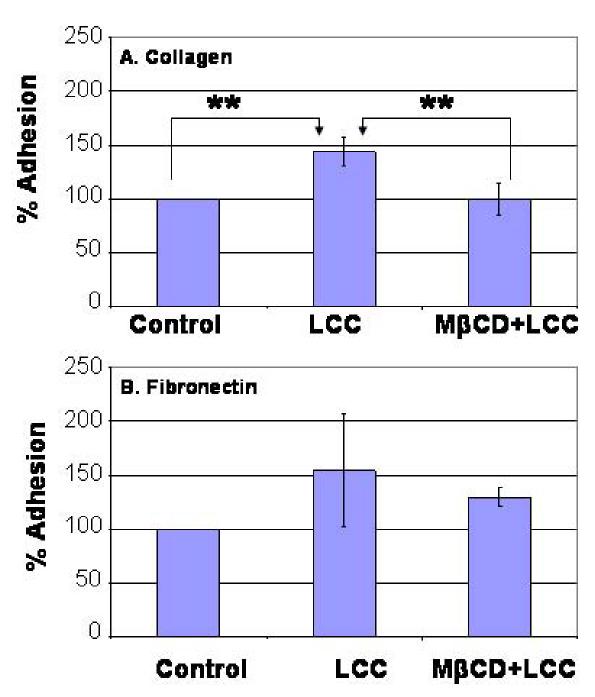


Fig. 4. Effect of LCC treatment on adhesion to collagen and fibronectin

Calcein AM-stained cells were treated with a lipoprotein-cholesterol concentrate (LCC; 57 mg/dl cholesterol) or M $\beta$ CD (15 mM) followed by treatment with LCC (M $\beta$ CD + LCC) prior to incubation on (A) collagen or (B) fibronectin surfaces. Adhesion was assessed as described in the text. The data are presented as percentage of adhesion of untreated cells (control), which was arbitrarily set to 100%. The level of adhesion to collagen after LCC treatment (LCC) was significantly different from that of control cells or that of cells treated with both reagents (n=3, \*\*P<0.01). The level of adhesion to collagen for cells treated with M $\beta$ CD followed by LCC (M $\beta$ CD + LCC) was not significantly different from that of control cells (P>0.05). None of

the adhesion values for trophozoites adhering to fibronectin surfaces were significantly different from each other (P>0.05).

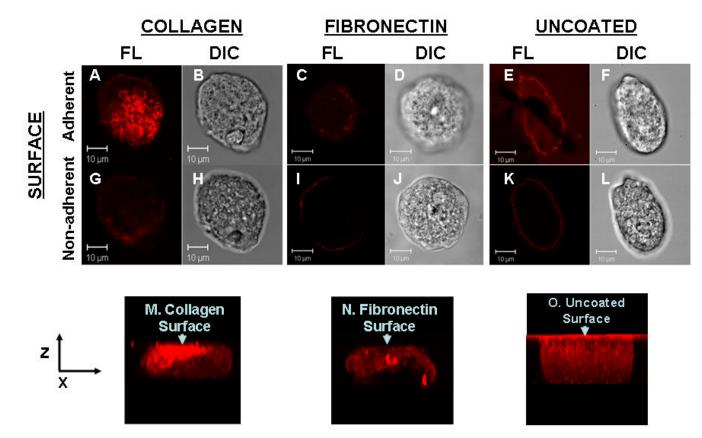
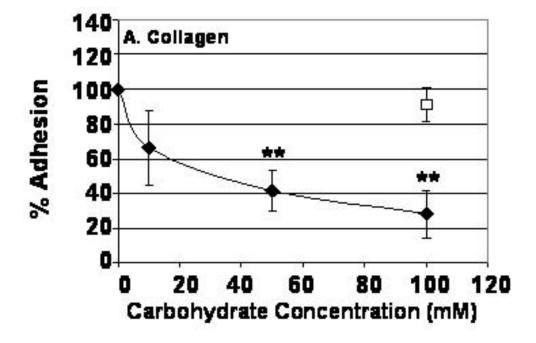


Figure 5. Lipid rafts are enriched at parasite-ECM interfaces

Fluorescence (FL) images of DiIC<sub>16</sub>-stained cells adhering to collagen-coated cover slips (A,B,G,H,M), fibronectin-coated cover slips (C,D,I,J,N) and uncoated cover slips (E,F,K,L,O). Lipid rafts are enriched at the surface of cells adhering to collagen (A) and, to a lesser extent, at the surfaces of cells adhering to fibronectin (C) or uncoated surfaces (E). Panels G,I, and K represent the non-adherent surface of the cells. Panels M-O are three-dimensional reconstructions viewed in the X-Z plane. The parasite-surface interface is depicted by the arrow. Corresponding differential interference contrast (DIC) images are also shown (B,D,F,H, J, L). Scale bars represent 10 µm.



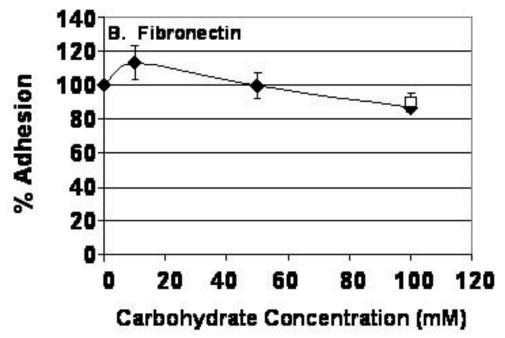


Figure 6. Galactose inhibits adhesion to collagen but not to fibronectin

Calcein AM treated cells were incubated in (A) collagen- and (B) fibronectin-coated 96-well plates in the presence of a range of concentrations of galactose (10-100 mM;  $\spadesuit$ ) or 100 mM mannose ( $\Box$ ). After washing off non-adherent cells, adhesion was quantified as described in the text. The data are expressed as a percentage of adhesion of untreated control cells, which was arbitrarily set to 100%. The data represent mean  $\pm$  S.D. of 3 trials for collagen and 4 trials for fibronectin. Galactose significantly inhibits adhesion to collagen in a dose-dependent fashion (\*\*P<0.01). Galactose does not significantly inhibit adhesion to fibronectin (P>0.05). Mannose exerts no significant effect on adhesion to either ECM component (P>0.05).