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Involvement of the actin cytoskeleton and p21^{rho}-family GTPases in the pathogenesis of the human protozoan parasite *Entamoeba histolytica*

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Abstract

It has been estimated that infection with the enteric protozoan parasite *Entamoeba histolytica* kills more than 50,000 people a year. Central to the pathogenesis of this organism is its ability to directly lyse host cells and cause tissue destruction. Amebic lesions show evidence of cell lysis, tissue necrosis, and damage to the extracellular matrix. The specific molecular mechanisms by which these events are initiated, transmitted, and effected are just beginning to be uncovered. In this article we review what is known about host cell adherence and contact-dependent cytolysis. We cover the involvement of the actin cytoskeleton and small GTP-binding proteins of the p21^{rho}-family in the process of cell killing and phagocytosis, and also look at how amebic interactions with molecules of the extracellular matrix contribute to its cytopathic effects.

Key words
- Amebiasis
- Actin cytoskeleton
- p21^{rho}
- Small GTPases
- Extracellular matrix

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Introduction

Entamoeba histolytica is a human enteric protozoan parasite that causes in excess of 40 million cases of colitis and liver abscess worldwide and results in more than 50,000 deaths annually. This makes amebiasis the third leading cause of death due to parasitic disease after malaria and schistosomiasis (1). There are two stages in the life cycle of the parasite. Infection occurs when the quadrinucleate cyst is ingested via fecally contaminated food or water. After traversing the stomach, the cyst undergoes one round of cell division and excysts in the bowel as eight amebic trophozoites. The trophozoite form of the organism is responsible for tissue destruction in amebiasis.

In the great majority of cases of symptomatic amebiasis, the disease presents as inflamed, ulcerative lesions in the colon. The most common secondary site of infection is the liver where considerable tissue damage can occur (2). Amebic colonic abscesses in humans are characterized by a relatively small number of amebae, usually staining for ingested erythrocytes, inhabiting the flask-shaped lumen of the abscess (3) (Figure 1).

Determining the prevalence of *E. histolytica* in the population is confounded by the existence of the non-pathogenic but morphologically identical *Entamoeba dispar* and *Entamoeba mushkovskii*. Distinguishing between them has required relatively sophisticated zymodeme (4) or ribosomal RNA (5) analysis, though methods based on species-specific monoclonal antibodies are now available (6,7). Together, the three *Entamoeba*

species are estimated to infect approximately half a billion people, including up to 20% of those living in the tropics and up to 5% of those in temperate regions (1,2).

Invasion of host tissues by the trophozoite is accompanied by contact-dependent cell-killing and phagocytosis. In the absence of attachment to host target cells, killing does not occur (8,9). The trophozoite form of *E. histolytica* is perhaps the most potent killing cell known. Tests assessing the cytolytic capacity of cytotoxic T lymphocytes use a range of effector to target cell ratios of 50:1 to 5:1 and an incubation time of at least 4 h (10). In contrast, assays of amebic killing use a range of ameba to target cell ratios of 1:5 to 1:50 and an incubation time that varies between 30 and 90 min (8,9,11). It should be noted, however, that a trophozoite of *E. histolytica* is on average forty times larger than a typical eukaryotic cell.

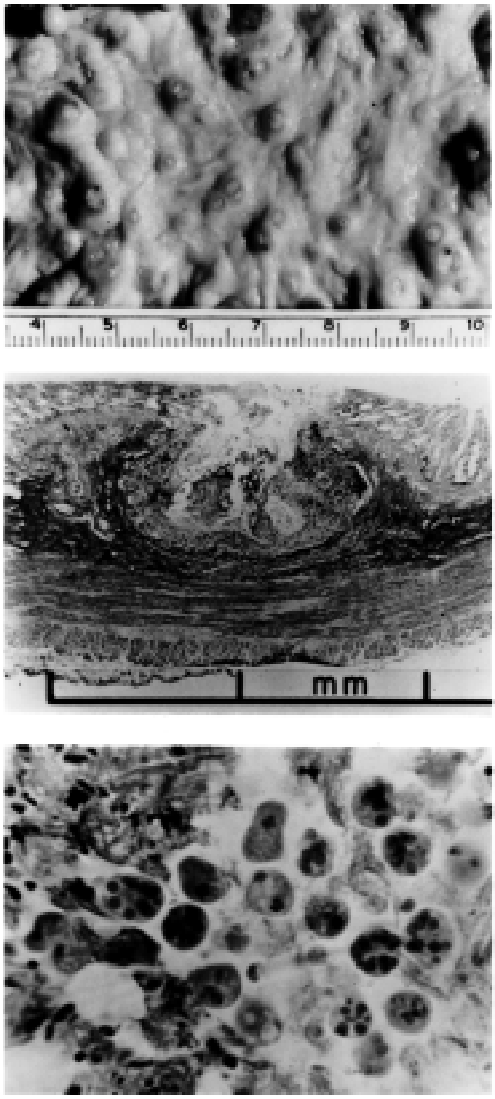

Figure 1 - Views of amebic abscesses of the colon. *Top panel*, Section of ulcerated colon from the luminal side. The tops of the abscesses contain the site of entry of the trophozoite. *Middle panel*, A cross-section of one of the flask-shaped ulcers in the colon. The amebic entry point is at the top of the ulcer which protrudes through the basal lamina into the submucosal lining of the colon. *Bottom panel*, This shows the amebae inhabiting one of the ulcers. In most of the trophozoites, ingested erythrocytes can be seen. This figure is composed of pictures from the personal collection of the late Harrison Juniper.

In addition to killing host cells, amebic invasion results in the degradation of the host's extracellular matrix (ECM) in the afflicted area (12). Binding of amebae to the ECM is accompanied by the directed secretion of proteases into the matrix *in vitro* (13). The molecular events involved in the initial recognition of host target cells and ECM by the ameba are just beginning to be characterized, though many of the proteins involved in adherence are known to some extent. These processes are critical to pathogenesis, and discerning their mechanism, besides answering questions basic to cell biology, should prove useful for the prevention and/or treatment of amebiasis. How signaling pathways in the amebae which lead to cytolysis and degradation of the host ECM might be activated by adherence to either host cells or matrix material is the subject of this review.

A Gal/GalNAc-specific lectin mediates attachment to target cells

Amebic adherence to host target cells is required for subsequent cytolysis and/or phagocytosis (8,9). The amebic molecule chiefly responsible for adherence to target cells is a lectin which mediates attachment to a wide variety of human cell types including colonic epithelium and lymphocytes (see 14 for review). This lectin binds specifically to galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) residues, and the addition of either sugar can prevent amebic attachment, and hence cytolysis, *in vitro* (9). The lectin consists of two subunits, one of 170 kDa and the other of 35/31 kDa, linked by disulfide bonds. The 31-kDa version of the small subunit is anchored in the membrane

by a glycosylphosphatidylinositol (GPI) lipid and the 170-kDa subunit has a single membrane-spanning region and a short (38 residue) cytoplasmic tail (15-20). The cytoplasmic tail displays some sequence identity with regions of some ß-integrins (21,22) and the epidermal growth factor receptor (23) that are involved in signaling.

The role of the Gal/GalNAc lectin in adherence has been further defined by specific monoclonal antibodies (mAbs). mAbs which recognize distinct epitopes of the heavy subunit of the lectin exert different effects upon the interaction of the amebae with target cells. Some of the antibodies inhibit adherence and cytolysis (11,24), but two activate the lectin by increasing adherence (24) and one of these actually decreases cell killing while enhancing cell adherence (11,24). mAbs specific for the light subunit of the lectin have no measurable effect on the adhesion or cytolysis of target cells (25). While it is well established that the lectin is responsible for binding of the ameba to most target cells, it is probably not the sole receptor mediating attachment (26). It is not clear if the signaling pathway that leads to cytolysis is initiated directly by the binding of the lectin, though the result with the enhancing monoclonal antibody certainly suggests that the two may be linked.

If the Gal/GalNAc-specific lectin does prove to be directly involved in the initiation of the cytolytic pathway, how might the effect of the lectin binding to a molecule on the target cell be transmitted to the amebae such that it is rendered capable of lysing the bound cell? Two variations of a model suggest themselves. The lectin itself, upon binding to a target cell, might undergo a conformational change which is transmitted to its cytoplasmic domain. As mentioned above, the cytoplasmic domain of the lectin heavy chain bears some resemblance to the cytoplasmic domains of known signaling molecules (21-23). This change in conformation would, through some mechanism, trigger a signal transduction cascade that ultimately leads to cytolysis of the bound cell - perhaps by a means akin to the perforin/granzyme B-based mechanism of T-cell mediated cytotoxicity (27). A second possibility for activation of a cytolytic signaling pathway involves the clustering of the lectin heterodimers upon binding to a target cell. Such a grouping of lectin molecules, analogous to integrin clustering (28,29), might bring together necessary factors for the triggering of cytolysis of the target cell. Considerable experimental work is needed in order to discriminate between these two possibilities.

Early events in parasite-host interaction: involvement of the actin cytoskeleton

What are the molecular events that immediately follow binding of the ameba to a target cell? Details are sparse, but localized actin polymerization around the site of contact is among the first events (30). Upon binding to either a red blood cell or a negatively charged liposome harboring galactose-containing glycoproteins/glycosphingolipids, an increase in cortical actin polymerization can be seen in the ameba at the site of contact within five seconds (Figure 2) (30-33). This correlates with the extension of an engulfing pseudopod. The polymerized actin content in a challenged ameba is double that of unchallenged cells, reaching this total within 4 min of adherence to target red blood cells (30). Actin polymerization can be inhibited by pre-incubating amebae with galactose or GalNAc before challenge (33), suggesting that the Gal/GalNAc lectin is involved in the initiation of actin polymerization. While latex beads are phagocytized by amebae, they do not stimulate an actin response, nor are they able to inhibit the interaction with red blood cells. Erythrocytes, however, diminish the phagocytosis of latex beads (30). These studies on amebic phagocytosis indicate that galactose- or GalNAc-containing

lipids or proteins can instigate a full phagocytic response (including mobilization of the actin cytoskeleton) in the amebae, when they appear in the context of negatively charged phospolipids. This work raises certain questions regarding molecular discrimination on the part of the amebae. Red blood cells are the only known human cells not subject to direct killing by amebae. While they must contain the motifs necessary for both binding and a phagocytic response on the part of the amebic trophozoite, they obviously lack something that is critical for effecting the cytolytic response.

Actin polymerization is essential for the cytolytic as well as the phagocytic activity of the ameba since treatment with cytochalasin D at 37°C abolishes both activities (8,9,30), and induces the amebae to become spherical. Cytochalasins block actin polymerization by binding to the fast-growing end of actin, but do not cause actin depolymerization. Agents (including cytochalasin, phorbol ester, and forskolin) that perturb the amebic cytoskeleton result in the up-regulation of actin at the transcriptional level (34). The actin cytoskeleton of ameba, when compared with that of mammalian cells, is quite disorganized and lacks stress fibers (35). Figure 3 shows the filamentous actin staining in a typical, motile ameba. All of the F-actin is concentrated in the leading pseudopod extended by the amebae, and no network of actin bundles that is seen in some mammalian cell types can be observed.

The actin cytoskeleton of the ameba and small GTPases of the p21^{rho} family

There is abundant evidence that the actin cytoskeleton of the ameba is vital for adherence to target cells and cytotoxicity as described above. The p21^{rho} (Ras homology) family of small GTPases is responsible for the formation and maintenance of specific

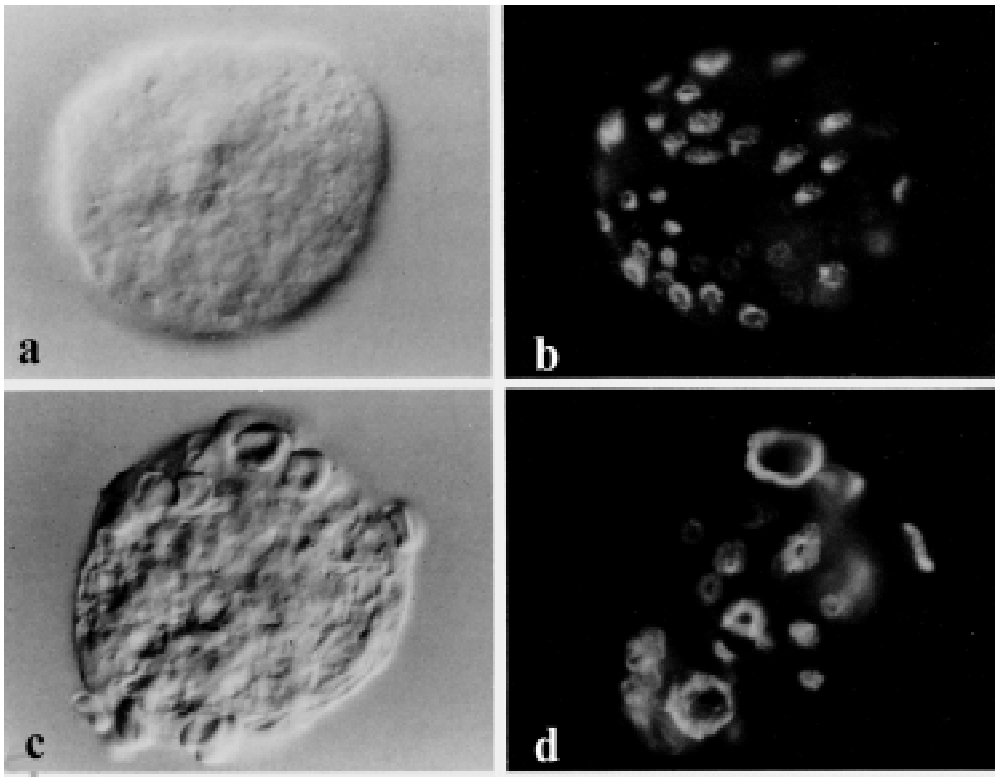

Figure 2 - Differential interference contrast (Nomarski) and corresponding fluorescence micrographs of glutaraldehyde-fixed, Triton X-100-extracted, and rhodamine-phalloidin trophozoites of *E. histolytica* before and after challenge with red blood cells. *Panels a and b*, Unchallenged ameba at 37°C exhibiting actin-lined macropinocytotic invaginations of varying size and number. *Panels c and d*, Ameba 5 s after challenge with red blood cells. Many of the attached erythrocytes were surrounded by polymerized actin. The pinocytic invaginations were still apparent. Reproduced from the *Journal of Experimental Medicine*, 1985, 162: 546-558 by copyright permission of The Rockefeller University Press and Dr. Gordon Bailey.

actin structures in cells from fungal to mammalian (36,37) among other roles (38). The $p21^{rho}$ family proteins, which include Rho, Rac, and Cdc42, act as molecular switches. When they are bound to GDP they are "inactive" and when bound to GTP they are "active". There are primarily two classes of molecules which regulate them. Guanine exchange factors (GEFs) promote the release of GDP and the binding of GTP and so lead to activation. GTPase activating proteins (GAPs) bind to small GTPases and enhance their latent GTPase activity, thus turning off the switch. Rho proteins have been linked to the regulation of cytolysis (39) and invasion (40-42) in mammalian cells. Rho and Rac proteins have been cloned from *E. histolytica* (43-45) (Godbold GD and Mann BJ, unpublished data).

Within the past year, studies have begun to appear on the role that Rho proteins play in the ameba. Two constitutively active forms of the Rac proteins have been expressed in the ameba, and they reveal intriguing differences. EhRacA (i.e., RacA from *Entamoeba histolytica*) apparently has a role in cell division and phagocytosis (46). Upon expression of the constitutively active RacA mutant, amebae take twice as long as normal to separate after cell division. They are also significantly defective in phagocytosis for bacteria, erythrocytes, and mucin-coated beads. The distribution and morphology of amebic actin also appears somewhat altered (46). Constitutively active EhRacG produces defects in cytokinesis when expressed in amebae, and also affects the regulation of the uroid (45, and Nancy Guillén, unpublished data). The uroid is a unique feature of the ameba and is formed by the actin cytoskeleton at the "rear" of the organism (47). This organelle, which generally appears as a singularity, is important in the elimination of capped surface proteins of the amebae by membrane shedding and is therefore thought to help the ameba avoid the host immune response. When constitutively active EhRacG is expressed in the amebae, multiple uroids develop and the cell becomes depolarized (45, and Guillén N, unpublished results).

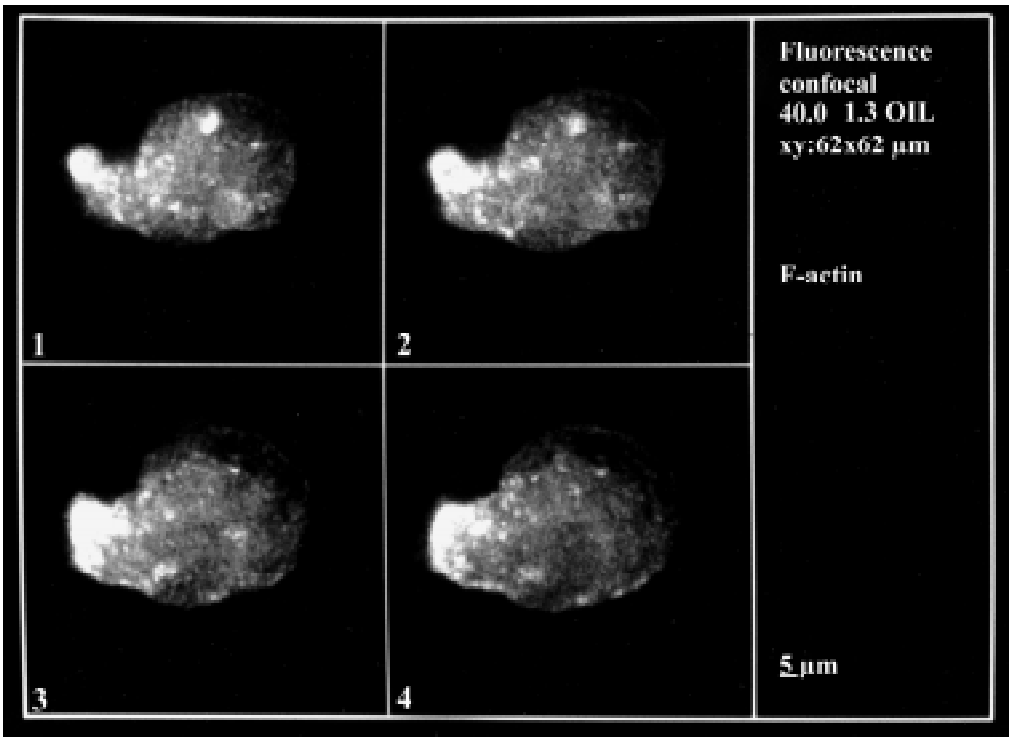

Figure 3 - Actin staining in the leading pseudopod of a trophozoite of *E. histolytica*. Trophozoites were seeded in PBS on an acetone-washed coverslip, fixed with paraformaldehyde and permeabilized with Triton X-100 before labeling amebic filamentous actin with Bodipy-phalloidin. The amebae were then subjected to confocal microscopy. The first panel shows a section closest to the coverslip upon which the ameba is fixed with successive panels 'ascending' through the ameba at intervals of 0.5 µm. Courtesy of Dr. Nancy Guillén.

The nature of the cytolytic response

As mentioned above, there is some evidence that adherence through the lectin can be at least partially decoupled from cytolysis. Monoclonal antibodies specific for one epitope of the Gal/GalNAc-specific lectin increase adherence upon binding to its epitope but decrease cytolysis (11). This suggests that the lectin is directly involved in the control of the cytolytic response of the ameba and that its role in adherence can be separated from its (putative) role as a signaling molecule.

Cytolysis can be increased to 200% of normal by treatment of trophozoites with phorbol 12-myristate 13-acetate (PMA) while adherence is unaffected (48). Since phorbol esters activate protein kinase C (PKC), it seems reasonable to suppose that PKC may play a role in either the induction or the propagation of the cytolytic response. Activation of PKC has also been shown to increase amebic adhesion to a fibronectin-coated surface, to instigate the release of proteases, and to boost levels of filamentous actin (49). In addition, treatment with phorbol esters induces a rearrangement of the actin cytoarchitecture resulting in an increase in amebic adhesion plates, structures which resemble metazoan focal contacts or focal adhesions (50). The changes (increases in adhesion, protease release, and levels of filamentous actin) induced by treatment of amebae with phorbol ester mirror the changes that follow treatment of amebae with fibronectin (49). Treatment with the PKC inhibitor H7 before stimulation with phorbol ester or fibronectin inhibits those effects (49), suggesting that PKC may lie distal to the amebic molecules that interact with fibronectin. Increased phosphorylation of amebic adhesion plate proteins is observed upon treatment with fibronectin or phorbol esters and is inhibited by H7, by the kinase inhibitor staurosporine, and by a pseudosubstrate of protein kinase C (50).

Amebic interaction with the extracellular matrix

Invasion of human tissues by *E. histolytica* involves a number of processes common to metastatic and phagocytic cells. This ability to invade is thought to be an evolutionarily conserved mechanism (for reviews see 51,52). The steps necessary for metastatic and amebic invasion include attachment to the target cell or to molecules of the extracellular matrix, and destruction of the molecules of the matrix - typically by proteolysis. Adherence to either molecules of the ECM or adjoining cells precedes proteolytic destruction of the matrix. This adherence is mediated by surface receptors on the invading cell, and their ligation is thought to trigger rearrangement of the cytoskeleton and the secretion of proteases. Trophozoites of *E. histolytica* have been shown to preferentially recognize and degrade ECM both *in vitro* and *in vivo* (13,53-55). This recognition and attachment has been specifically demonstrated in the case of amebic fibronectin receptors, some of which resemble metazoan ß-integrins (56,57). Digestion of collagenous matrix by trophozoites has also been demonstrated and is believed to be similarly controlled by cell surface receptors (58). The amebae possess a class of cysteine proteinases which have a notable binding affinity for laminin (59). Attachment of the amebae to components of the ECM triggers the formation of adhesion plates with accompanying changes in the actin cytoskeleton (49,50,56). Since adherence is a prerequisite for both ECM destruction and cell killing, it is reasonable to suppose that binding of an ameba through its surface receptors induces signals by which degradation of the ECM and cytolysis of the target host cells are effected, though probably through signaling pathways that are independent of one another.

The binding of trophozoites to molecules of the ECM leads to the formation of amebic

adhesion plates (13). Amebic adhesion plates contain several proteins similar to those found in mammalian focal adhesions or focal contacts including actin, the actin binding proteins α-actinin, vinculin and tropomyosin, myosins I and II and a protein similar to pp125 focal adhesion kinase (FAK) (50). Binding of amebae to the ECM can be mediated by a 37-kDa receptor protein specific for fibronectin (13,56). A 140-kDa protein that is similar to the mammalian fibronectin-binding protein, $ß_1$ integrin, has also been found in the ameba (57). Focal adhesions in mammalian cells are formed at the plasma membrane and serve to anchor the cell to the ECM through integrin heterodimers. They are the point of termination for bundles of filamentous actin known as stress fibers which provide structural integrity and resistance to mechanical forces. In addition, focal adhesions serve as signaling organelles, carrying information from the ECM to the cell (60). While amebae have no obvious stress fibers (35) numerous investigations have shown that they have signaling pathways similar to those of metazoan cells.

PKC has been found to translocate to adhesion plates upon stimulation of amebae with phorbol esters or fibronectin (49). Pharmacological inhibitors of PKC can block the phosphorylation of adhesion plate proteins that normally follows interaction with fibronectin (49). The interaction of amebae with proteins of the ECM results in local degradation at the site of contact between trophozoites and the substrate (13). This degradation has been correlated with the formation of adhesion plates (56) and it is thought that the formation of the plates may orient the secretion of proteases.

Stimulation of trophozoites with collagen I results in the autophosphorylation of FAK, and it is speculated that the interaction of amebic integrin-like proteins with other membrane proteins or cytoskeletal components might activate amebic FAK (58). The FAK-like protein is tyrosine phosphorylated in response to the ameba binding to collagen (58) as well as a molecule immunologically similar to mitogen-activated protein kinase (MAPK), suggesting the existence of a signaling pathway leading from the extracellular matrix. This specific phosphorylation of FAK is seen as early as 15 min after stimulation with collagen and Ca^{2+} and peaks at 60 min (61). FAK is one of the principal kinases participating in signaling mediated by integrins from focal adhesions (60). Interestingly, Rho proteins are critical in the formation of focal adhesions in mammalian cells (62,63) as well as in the signaling to and from focal adhesion (60,64,65).

In summary, understanding the signal transduction pathways involved in the pathogenic activity of the protozoan parasite *E. histolytica* may unearth new ways in which it can be controlled. The role of the Gal/GalNAc-specific lectin in the induction of a cytolytic response remains to be elucidated as well as the mechanism of that response - does it resemble the perforin/granzyme B-based mechanism of T-cell mediated cytotoxicity? What function does actin play in cytolysis and in the direction of protease secretion during invasion of the trophozoite? Do Rho family proteins have a place? The interaction between the ameba and the extracellular matrix has been the best characterized of all these phenomena, but there are far more questions than answers still, and many intriguing directions for further research.

The study of the molecular mechanisms responsible for the attachment of this deadly parasite to target cells and the ECM has advanced steadily over the past generation, but has been limited, until recently, by the inability to manipulate the genome via DNA transfection techniques. Now that this obstacle has been overcome (66-69), more detailed investigations into the molecular mechanisms of attachment, death, and destruction can be conducted. With the abundance of amebic proteins that have been

cloned in the last few years, one looks forward with considerable anticipation to the future *in vivo* studies of their role in the pathogenesis of *Entamoeba histolytica*.

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