

# Interaction between Pathogenic Amebas and Fibronectin: Substrate Degradation and Changes in Cytoskeleton Organization

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**Abstract.** Invasion of human tissues by the parasitic protozoan *Entamoeba histolytica* is a multistep process involving, as a first step, the recognition of surface molecules on target tissues by the amebas or trophozoites. This initial contact is followed by the release of proteolytic and other activities that lyse target cells and degrade the extracellular matrix.

In other parasitic diseases, as well as in certain cancers, the interaction of invasive organisms or cells with fibronectin (FN) through specific receptors has been shown to be the initial step in target cell recognition. Interaction with FN triggers the release of proteolytic activities necessary for the effector cell migration and invasion. Here, we describe the specific interaction of *Entamoeba histolytica* trophozoites with FN, and identify a 37-kD membrane peptide as the putative receptor for FN. The interaction between the parasite and FN leads to a response reaction that includes the secretion of proteases that degrade the

bound FN and the rearrangement of amebic actin into "adhesion plates" at sites of contact with FN-coated surfaces. The kinetics of the interaction was determined by measuring the binding of soluble <sup>125</sup>I-FN to the trophozoites and visualization of the bound protein using specific antibodies. Degradation of FN was measured by gel electrophoresis and the release of radioactivity into the incubation medium. Focal degradation of FN was visualized as black spots under the trophozoites at contact sites with fluorescent FN.

We conclude that the interaction of *E. histolytica* with FN occurs through a specific surface receptor. The interaction promotes amebic cytoskeleton changes and release of proteases from the parasite. The binding and degradation of extracellular matrix components may facilitate the migration and penetration of amebas into tissues, causing the lesions seen in human hosts.

**T**HE capacity of invasive cells to penetrate solid organs is considered to be a multiple step process requiring intimate surface contact between effector cells and targets. This interaction may take place through specific receptor-mediated mechanisms which result in the lysis of target cells or substrates. Cell lysis can be accomplished by release of proteases and other lytic mediators and by the insertion of pore-forming proteins into the plasma membrane of the target cell (for a review see reference 39). In addition to causing cell lysis, invasive cells can attach to and destroy extracellular matrix components during migration through connective tissue barriers (15, 24), as is the case with macrophages (35), endothelial cells (16), and the implantation of trophoblasts cells during mammalian embryogenesis (11). Fibronectin-degrading proteases have also been reported in invading cancerous cells (8, 15).

*Entamoeba histolytica* trophozoites are invasive cells capable of tissue penetration and intestinal ulcer formation in human hosts (22). The mechanisms of invasion are not well understood, although close parasite-target cell interac-

tion seems to be a requirement (19, 25, 38). Degeneration of intestinal epithelial cells adjacent to invading trophozoites and dissolution of the basement membrane of the mucosa have been observed (32). These findings suggest that histolytic factors play a role in invasion by the parasite. Many lytic activities have been identified in *E. histolytica*, either membrane bound or released into the medium (10, 17, 21), including a pore-forming protein that is thought to assemble itself in the target cell membrane (19, 38). During the invasive process of this parasite, besides contact-dependent killing of host cells, some of the major components of the extracellular matrix such as collagen, laminin, and fibronectin have been shown to be degraded (4, 17, 23, 32). As components of the extracellular matrix are also targets for the battery of proteolytic enzymes present in invasive amebas, their destruction would allow the trophozoites to invade the large intestine and subsequently other organs such as the liver, lungs, brain, etc.

Fibronectin (FN)<sup>1</sup> is known to bind to a variety of com-

1. Abbreviation used in this paper: FN, fibronectin.

ponents, including proteoglycans, collagen, glycosaminoglycans, actin, and glucose (14). FN also binds to the surface of many eukaryotic and prokaryotic cells. In eukaryotic cells this interaction produces changes in cell morphology and in the cytoskeleton (13, 14, 29, 37). Specifically, high levels of fibronectin induce formation of organized arrays of actin microfilaments, possibly through a transmembrane linkage (29). Fibronectin receptors have been isolated and characterized in several types of cells (2, 28, 31). In certain bacteria a FN receptor is thought to play a role in adherence to epithelial cells (1, 9). *Trypanosoma cruzi* (26, 28), *Plasmodium falciparum* (27), and *Leishmania* (36) (all intracellular protozoan parasites) also have FN-receptors. The presence of a FN receptor in these organisms may aid in the initial interaction of these pathogens with host cells and tissues. A FN receptor on the surface of *E. histolytica* trophozoites may also be required for their penetration through tissues. We report here the specific binding of *E. histolytica* trophozoites to FN and the identification of a putative cell surface receptor for this protein. Moreover, we find that contact with FN triggers major rearrangements of the *E. histolytica* cytoskeleton and leads to the secretion of proteases by the trophozoites.

## Materials and Methods

### Cell Culture

*Entamoeba histolytica* HM-1:IMSS, clone BX1, and *Entamoeba histolytica* Laredo-type trophozoites were grown in TYI-S-33 medium as described by Diamond et al. (7). Amebas for all the experiments were harvested during logarithmic growth by centrifugation at 200 g. Cell pellets were washed several times with 0.9% NaCl, 0.005 M Tris-HCl, pH 7.4.

### Fibronectin Preparation

FN was purified by a modification of the gelatin-Sepharose affinity chromatography method (30) from fresh human blood collected in  $10^{-4}$  M phenylmethylsulfonyl fluoride, and 5% sodium citrate.

Protein purity was monitored in 5% SDS-discontinuous polyacrylamide gels. The purified FN was dialyzed against 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, and stored at  $-70^{\circ}\text{C}$ . Purified plasma FN was quantified using an extinction coefficient of 1.28 at 280 nm.

### Preparation of Antifibronectin Antibodies

Fibronectin antibodies were prepared by immunizing rabbits with two intramuscular doses of 150  $\mu\text{g}$  FN in Freund's complete adjuvant (Difco Laboratories, Detroit, MI) followed 2 wk later with 500  $\mu\text{g}$  FN, and a final intravenous boost 1 wk later with 300  $\mu\text{g}$  FN without adjuvant. Antibodies were purified by passing the serum through a FN affinity column, eluting with 0.2 M glycine pH 2.8, and collecting 500- $\mu\text{l}$  fractions in tubes containing 100  $\mu\text{l}$  of 1.0 M Tris-HCl pH 8.6. Fractions containing the antibody were pooled and dialyzed against PBS at  $4^{\circ}\text{C}$ , aliquoted, and stored at  $-70^{\circ}\text{C}$ . Final concentration of the antibody was 0.635 mg/ml.

### Indirect Immunofluorescence

Trophozoites were resuspended in PBS and incubated for 15 min in suspension with FN (0.2 mg/ml) in 0.15 M NaCl, 0.05 M Tris-HCl pH 7.4. Incubation was at  $4^{\circ}\text{C}$  to reduce the extensive FN degradation observed at higher temperatures. The cell suspension was then applied to a cushion of 100  $\mu\text{l}$  of dibutylphthalate (6) and centrifuged at 12,000 g. The pellet was washed once with 1% FN-depleted serum in PBS and cells fixed with 3% paraformaldehyde in PBS for 30 min. After fixation the cells were washed with the FN-depleted serum and centrifuged at 200 g. Rabbit anti-human FN (diluted 1:50 in PBS) was then added, and the cells were incubated for 30 min at room temperature. After washing the cells with PBS they were incubated for 30 min with a 1:20 dilution of the second antibody, fluorescein-conjugated sheep anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, PA). After three washes with PBS, the cells were mounted in PBS-glycerol.

### Fibronectin Iodination

100  $\mu\text{g}$  of FN (1 mg/ml) were radioiodinated by the Chloramine-T procedure (12) using 1 mCi of  $^{125}\text{I}$  (Amersham Corp., Arlington Heights, IL).  $^{125}\text{I}$ -FN was separated by chromatography on a Sephadex G-25 column equilibrated with 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4, and 10% BSA. The protein peak was pooled, aliquoted, and stored at  $-70^{\circ}\text{C}$ . The specific activity of the  $^{125}\text{I}$ -FN was 1.28  $\mu\text{Ci}/\mu\text{g}$ .

### Cell Attachment Assay

Microelisa plates (Dynatech Corp., Baton Rouge, LA) were incubated overnight at  $4^{\circ}\text{C}$  with 40  $\mu\text{l}$  of FN (250  $\mu\text{g}/\text{ml}$ ). The plates were then rinsed three times with PBS and incubated with the amebas (0.25  $\times 10^6/\text{ml}$ ) in 150  $\mu\text{l}$  of serum-free TYI-S-33 medium for different times at  $37^{\circ}\text{C}$ . At the end of the incubation, the nonadhered cells were recovered by aspiration with a Pasteur pipette and counted in a Coulter counter (Coulter Electronics, Hialeah, FL).

### Determination of Proteolytic Activity

Tissue culture multi-well plates (Linbro, Flow Laboratories, Hamden, CT) were coated with 30,000 cpm/well of  $^{125}\text{I}$ -FN by incubation overnight at room temperature under UV light. The plates were rinsed with 0.15 M NaCl, 5 mM Tris-HCl, pH 7.4, and incubated with the amebas (*E. histolytica*: 0.05  $\times 10^6/100$   $\mu\text{l}$ ; *Laredo*: 0.125  $\times 10^6/100$   $\mu\text{l}$ ) in the same buffer. The release of  $^{125}\text{I}$ -FN was determined by collecting the incubation medium at various time periods after trophozoite attachment and measuring radioactivity in a auto-gamma scintillation spectrometer (No. 5230; Packard Instrument Co., Inc., Downers Grove, IL). Control wells without amebas were also processed for each time point.

### Binding of $^{125}\text{I}$ -FN to *Entamoeba histolytica* and *Laredo* Trophozoites

*E. histolytica* ( $2 \times 10^6$ ) and *Laredo* ( $5 \times 10^6$ ) cells were incubated for different time periods in 80  $\mu\text{l}$  of 0.9% NaCl in 0.005 M Tris HCl pH 7.4 with different concentrations of  $^{125}\text{I}$ -FN in a total volume of 200  $\mu\text{l}$  at room temperature. 50  $\mu\text{l}$  of the mixture were applied to a cushion of 100  $\mu\text{l}$  of dibutylphthalate in a 400  $\mu\text{l}$  Eppendorf tube and centrifuged 1 min in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, NY). The tips of the tubes were cut with a razor blade and directly counted for radioactivity of the pellets.

### Preparation of Thin Sections for Autoradiography

PBS-washed trophozoites ( $1 \times 10^6/\text{ml}$ ) were resuspended in PBS and 100  $\mu\text{l}$   $^{125}\text{I}$ -FN were added. The cell suspension was incubated for 15 min with gentle shaking and the cells layered on top of a cushion of dibutylphthalate and centrifuged. Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at room temperature for 30 min, postfixed with 1%  $\text{OSO}_4$  in the same buffer for 30 min, and embedded in Epon 812. 0.5- $\mu\text{m}$  thick sections were mounted on glass slides and covered with Ilford K-5D emulsion diluted 1:1 with distilled water. The slides were exposed at  $4^{\circ}\text{C}$  for 2 wk, developed in Microdol X (Eastman Kodak Co., Rochester, NY), and stained with alkaline toluidine blue.

### Subcellular Fractionation of *Entamoeba histolytica* Trophozoites

Subcellular fractionation of trophozoites was carried out by the method of Aley et al. (3), using a cocktail of protease inhibitors containing 2 mM diisopropylfluorophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 2  $\mu\text{M}$  leupeptin, and 5 mM *N*-ethyl-maleimide in 10 mM Tris-HCl pH 7.5. The fractions obtained by differential centrifugation in mannitol-sucrose gradients and a 20% sucrose cushion were: plasma membranes, internal membranes, nonvesiculated membranes, and soluble components.

### Preparation of Crude Membranes

Cell pellets were washed with PBS and resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5) containing the protease inhibitors mentioned above. After homogenization, the homogenate was centrifugated at 150,000 g for 30 min and pellets used as crude membranes after resuspension in 10 mM Tris-HCl, pH 7.5.

## PAGE and Autoradiography

5 and 10% discontinuous SDS gels and 5–15% polyacrylamide gradient gels were run as indicated by Laemmli (18). To avoid proteolysis, the amebic protein samples were boiled before addition of SDS and sample buffer (24). Gels were fixed, stained with Coomassie Blue, and dried. Autoradiography was performed using X-OMAT Kodak x-ray film.

## Electroblot and Overlay

Immediately after the electrophoretic run the proteins were transferred at 60 V for 12 h to nitrocellulose membranes. The filter was blocked with 3% BSA, 0.1% Tween 20, and 0.1% sodium azide in PBS at pH 7.4 for several hours, and incubated overnight with 1.5 ml of  $^{125}\text{I}$ -FN (sp act, 0.9  $\mu\text{Ci}/\mu\text{g}$ ) diluted in 15 ml of 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4 at room temperature. The membranes were exhaustively washed with several changes of the same buffer containing 0.1% Triton X-100. Amebic proteins that bound  $^{125}\text{I}$ -FN were detected by autoradiography of the nitrocellulose membranes.

## Results

### Binding of $^{125}\text{I}$ -FN to *Entamoeba histolytica* and *Laredo* Trophozoites

The kinetics of binding of  $^{125}\text{I}$ -FN was studied with both *E. histolytica* and *Laredo* trophozoites. *Laredo* was used as a control because it is a noninvasive ameba. It is inefficient in lytic activities characteristic of *E. histolytica*, such as collagenase (23), contact lysis of target cells (25), and erythrophagocytosis (33). *E. histolytica* and *Laredo* trophozoites bound  $^{125}\text{I}$ -FN in a time-dependent manner (Fig. 1 A). For *E. histolytica* most of the reaction took place during the first 20 min. At 15 min *E. histolytica* bound 50% of the total FN bound in 1 h. The *Laredo* amebas bound FN more slowly and less effectively, and bound only 57% of the total protein bound by *E. histolytica* at the end of the incubation period. The saturation of the receptor with the ligand was analyzed by incubating a fixed number of trophozoites with increasing concentrations of  $^{125}\text{I}$ -FN. Fig. 1 B shows that the binding of *E. histolytica* reached saturation with 350 ng of radioactive FN. The highest concentration of FN tested for a given number of *Laredo* amebas was not saturating. Unlabeled FN efficiently competed with the radiolabeled ligand for binding to the amebas, although with *E. histolytica* only 70% inhibition was obtained at concentrations as high as 80  $\mu\text{g}/\text{ml}$  (Fig.

1 C) of unlabeled FN. This is probably due to the degradation of the ligand by the parasite, as will be shown below (Fig. 4). With *Laredo*, where there is no degradation of the bound FN, the unlabeled ligand totally inhibited the binding of the  $^{125}\text{I}$ -FN to the trophozoites (Fig. 1 C). There was no effect on the binding of  $^{125}\text{I}$ -FN when 100  $\mu\text{g}/\text{ml}$  of BSA, alkaline phosphatase, cocarboxylase, or cytochrome c, were used as competitors (data not shown).

### Localization of Bound Fibronectin in Trophozoites

The binding of FN to trophozoites was qualitatively assayed by indirect immunofluorescence. Live trophozoites, either *E. histolytica* or *Laredo*, were incubated with FN antibodies and then with a second FITC-labeled antibody. Several surface fluorescence patterns were observed (Fig. 2). Antibodies were distributed over the entire surface of the amebas, as irregular patches, or at times as a cap on one side of the cell.

### Identification of a Putative FN Receptor

To identify a FN receptor or a peptide with high affinity for FN in the trophozoites, solubilized subcellular fractions were analyzed by electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters. The filters were then incubated with  $^{125}\text{I}$ -FN and the FN-binding proteins detected by autoradiography. Fig. 3 B shows that all membrane fractions, plasmatic, internal, and nonvesiculated (lanes 1–5), contained a single 37-kD protein with FN-binding activity. The fraction corresponding to soluble components (Fig. 3, A and B, lanes 6 and 6') that contains mostly cytoplasmic proteins did not show the activity. Moreover, when nitrocellulose filters containing membrane fractions were incubated with unlabeled FN (100  $\mu\text{g}/\text{ml}$ ) there was complete inhibition of the  $^{125}\text{I}$ -FN binding to the 37-kD peptide (data not shown). These results suggest that the putative 37-kD FN receptor is a membrane component. Its binding activity appears to be very high because the peptide is not one of the major components of the membrane preparations as seen by the Coomassie stain of the separated proteins (Fig. 3 A). Binding of FN to the 37-kD peptide was also demonstrated by incubating membrane fractions with cold FN followed by the FN antibody and a second antibody coupled to

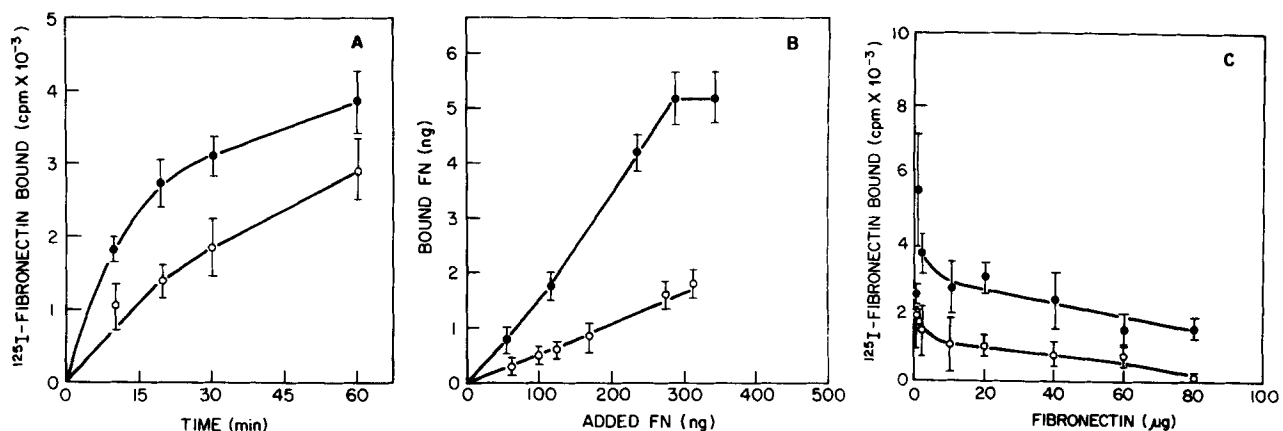
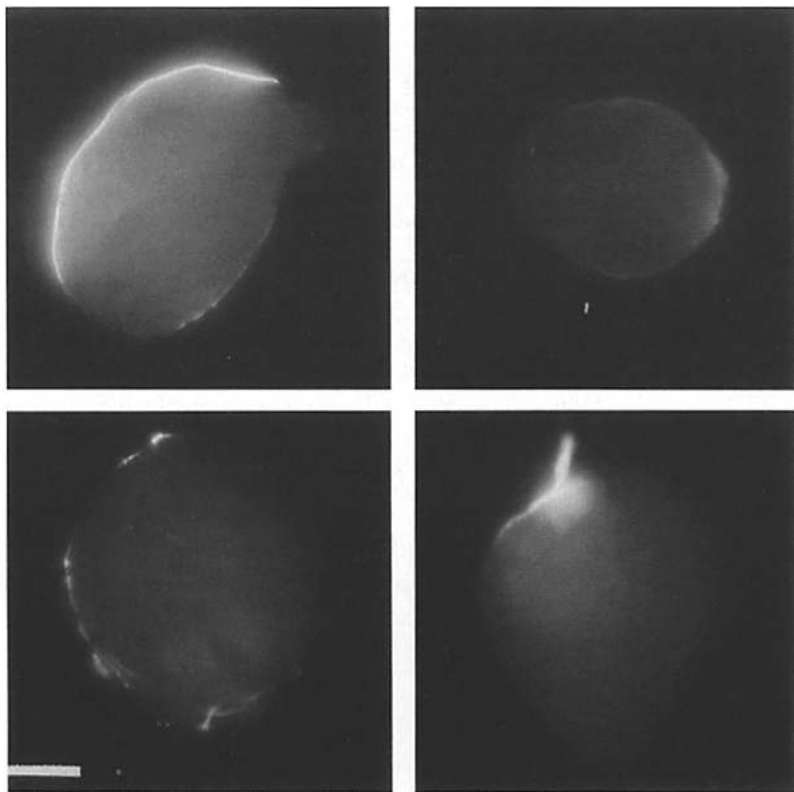


Figure 1. Characterization of FN binding to trophozoites. (A) Kinetics of binding of  $^{125}\text{I}$ -FN to *E. histolytica* ( $2 \times 10^6$  cells/200  $\mu\text{l}$ , ●), and *Laredo* ( $5 \times 10^6$  cells/200  $\mu\text{l}$ , ○). (B) Binding in the presence of increasing concentrations of  $^{125}\text{I}$ -FN. Incubation was for 15 min. (C) Competition between  $^{125}\text{I}$ -FN and unlabeled FN. 15  $\mu\text{g}/\text{ml}$  of  $^{125}\text{I}$ -FN were added together with increasing amounts of unlabeled fibronectin. Bars represent standard deviation of two separate experiments.



**Figure 2.** Visualization of bound FN to the trophozoite surface. After incubation with soluble FN for 15 min the cells were fixed and reacted first with antifibronectin antibody, then with a second antibody labeled with FITC. Cells viewed by epifluorescence show different distribution of antibodies on the surface. Bar, 10  $\mu$ m.

peroxidase. The positive band corresponding to the 37-kD peptide is shown in plasmatic (lane 1) and crude membrane (lane 2) fractions in Fig. 3 C.

#### **Fibronectin Degradation by *E. histolytica* Trophozoites**

The following experiment was performed to determine the integrity of the FN that remained associated with the cells after the incubation for different lengths of time. After incubation with  $^{125}$ I-FN the cells were solubilized and analyzed by electrophoresis on acrylamide gels (Fig. 4 A). It can be seen that the FN was progressively degraded into several peptides. The distribution of the radioactive peptides associated with the amebas was determined by fixing cells after 15 min of interaction with  $^{125}$ I-FN and processing for autoradiography. As can be seen in Fig. 4 B, silver grains were mostly localized in the cytoplasm associated with digestive vacuoles, indicating internalization of the bound protein. On the other hand the  $^{125}$ I-FN bound to *Laredo* trophozoites was not degraded (Fig. 4 C).

#### **Attachment to and Degradation of FN-coated Surfaces**

Data on binding of soluble FN to *E. histolytica* indicated not only specific binding of the protein to the trophozoites but also the release of degraded FN peptides into the medium. We measured the adhesion of amebas to fibronectin-coated and uncoated surfaces to corroborate these results and to study the mechanism of adhesion. It was found that 80% of amebas attached to FN-coated surfaces while only 20–25% bound to uncoated plastic or glass. In the presence of FN, adhesion was rapid, reaching a maximum between 15 and 20 min (Fig. 5 A). This is in agreement with the time course of soluble FN binding (Fig. 1 A). After maximum binding was

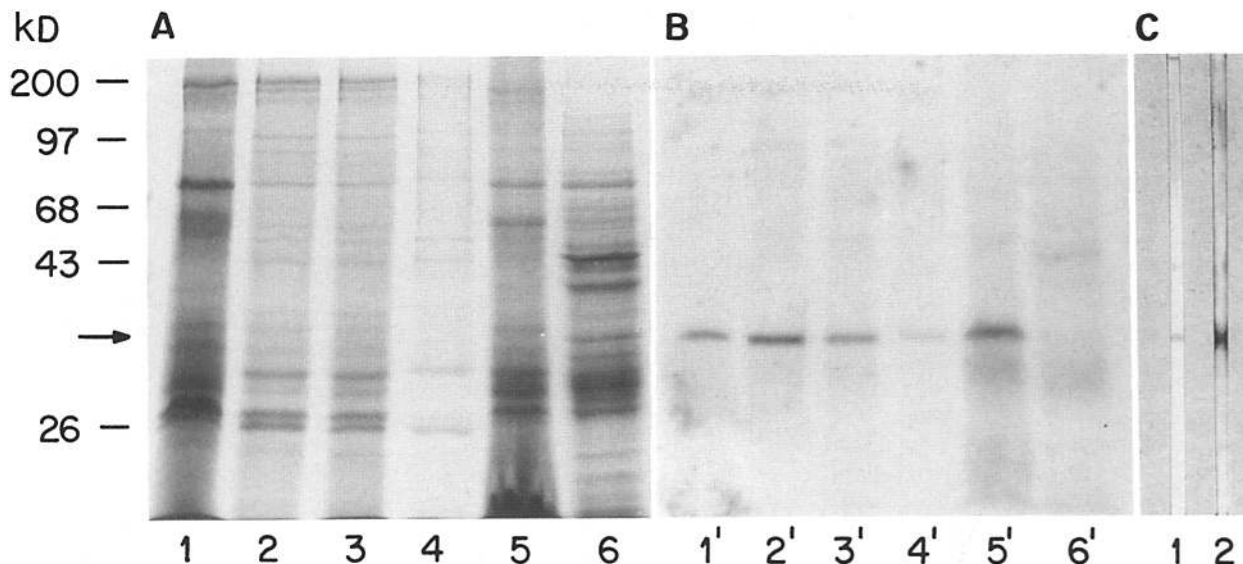
reached, the number of bound amebas decreased with time (Fig. 5 A). Adhesion to plastic was maintained at low levels, similar to the adhesion levels found with nonspecific substrates such as BSA.

The efficient binding of the amebas to FN-coated surfaces followed by the detachment as a function of time, suggested that cells may digest the FN substrate and thus be released. To test this hypothesis, amebas were allowed to attach and interact with FN-coated glass slides for 15 min. The slides were then washed and stained with an anti-FN antibody. As shown in Fig. 5 B, regions where cells were attached show loss of FN staining (black holes), presumably due to degradation of FN by the cells. *Laredo* amebas did not produce the black holes after interaction with the FN.

To corroborate that degradation of FN actually occurs, amebas were incubated on  $^{125}$ I-FN-coated plates and the release of radioactivity into the incubation medium was measured (Fig. 5 C). It can be seen that *E. histolytica* releases radioactivity into the medium with a time course similar to that of the binding of soluble  $^{125}$ I-FN to the cells and that of the attachment of cells to FN-coated surfaces. The released label in control wells, without amebas, never reached >15% of that released by *E. histolytica*. Very different results were obtained with *Laredo* trophozoites. Release of label after 120 min of interaction was only 17% as efficient as for *E. histolytica* (Fig. 5 C). This result confirms that *Laredo* lacks the activity(ies) necessary for FN degradation, although the protein binds to trophozoites (Fig. 1).

#### **Changes in the Distribution of Polymerized Actin Induced by FN Binding**

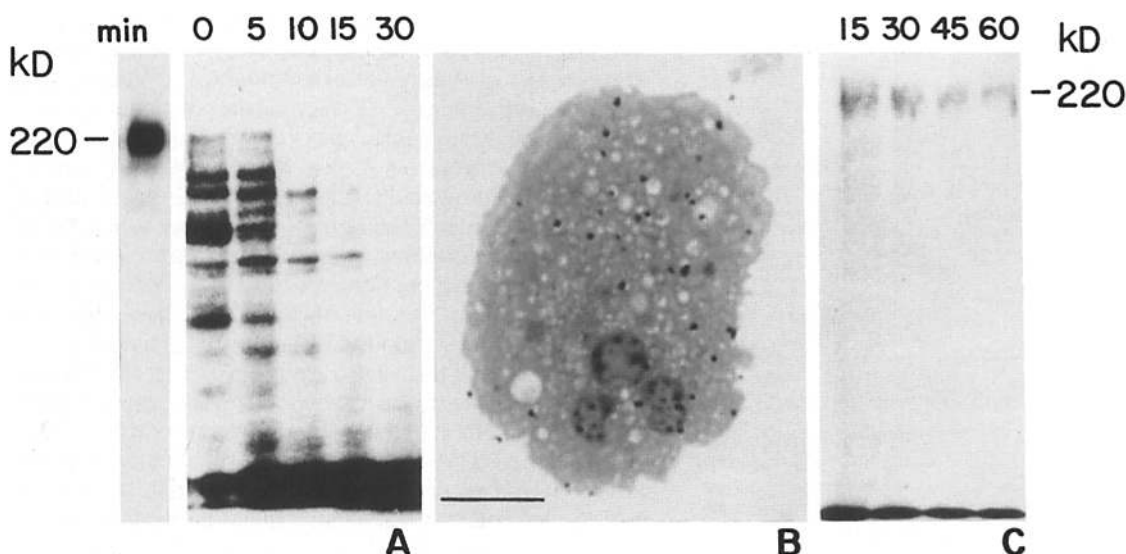
Changes in the cytoskeleton induced by binding of the amebas to different substrates were analyzed by staining of



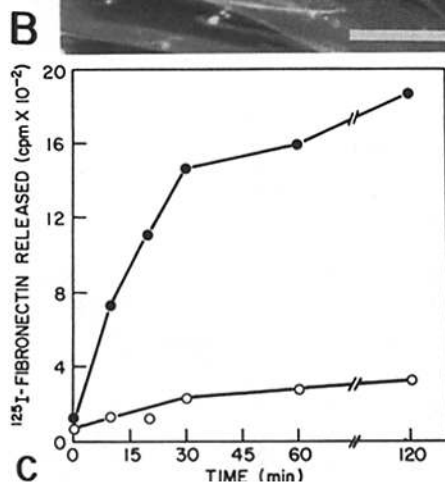
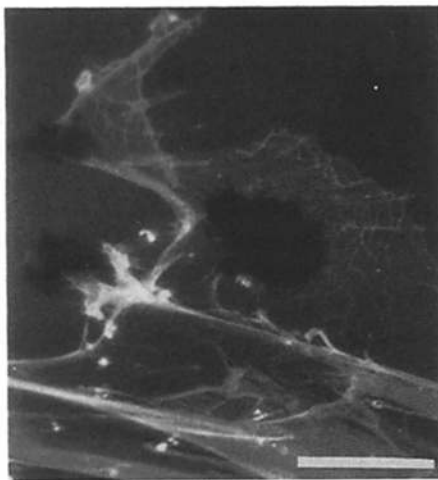
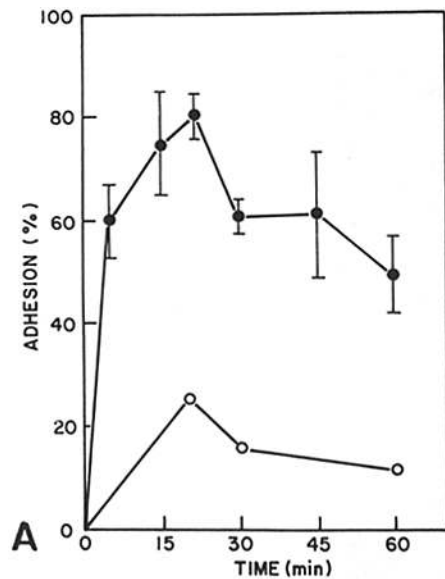
**Figure 3.** Identification of a FN-binding peptide. Subcellular fractions from amebas were prepared, solubilized, and fractionated by electrophoresis on 10% acrylamide gels. The lanes represent: (1) crude membranes; (2 and 3) plasma membranes in duplicate; (4) nonvesiculated membranes; (5) internal membranes; and (6) soluble components. Two gels were run in parallel, one (A) was stained with Coomassie Blue after electrophoresis. The proteins from the other (B) were transferred to a nitrocellulose filter and incubated with  $^{125}\text{I}$ -FN. (C) Blots of gels containing plasma and crude membrane fractions (lanes 1 and 2) were incubated first with unlabeled FN and then with anti-FN antibody and a second antibody coupled to peroxidase. The reactive band is shown. Arrow indicates the position of the 37-kD band identified by autoradiography or the FN antibody.

the polymerized actin with fluorescently tagged phalloidin. Amebas were cultured on three different substrates: FN-coated glass, BSA-coated glass, and uncoated regular glass. Fig. 6. (A and B) shows that the interaction with FN induced the polarization of amebic actin to form special structures at the interface with the FN-coated surfaces, reminiscent of

adhesion plates. These structures were not seen when amebas interacted with substrates such as BSA (Fig. 6 C) or uncoated glass (Fig. 6 D). Other types of actin-containing structures outlined by rhodamine-phalloidin correspond to phagocytic and pinocytic invaginations present in axenically cultured trophozoites (5). These invaginations (Fig. 6, ar-



**Figure 4.** Degradation and internalization of bound FN. (A) Autoradiography of the peptides derived from  $^{125}\text{I}$ -FN associated with *E. histolytica* trophozoites. Amebas were incubated with soluble  $^{125}\text{I}$ -FN for the indicated time. The cells were pelleted, washed, and dissolved in electrophoresis buffer and analyzed on 5–15% gradient polyacrylamide gels. The first lane contains the  $^{125}\text{I}$ -FN used for the experiment. (B) Thin section autoradiography of cells incubated for 15 min with soluble  $^{125}\text{I}$ -FN. Silver grains are mainly associated with digestive vacuoles. Bar, 10  $\mu\text{m}$ . (C) Autoradiography of a 5% polyacrylamide gel showing  $^{125}\text{I}$ -FN after the indicated periods of incubation with *Laredo* trophozoites. Incubation conditions were the same as for *E. histolytica*.



**Figure 5.** Attachment of trophozoites to solid substrates. (A) Time course of *E. histolytica* trophozoite attachment to fibronectin-covered and plastic surfaces. Cells ( $0.25 \times 10^6/\text{ml}$ ) in serum-free medium were incubated at  $37^\circ\text{C}$  in microelisa plates coated with FN (●) or uncoated (○). The cells that did not adhere were recovered and counted. (B) Visualization of the degraded fibronectin under *E. histolytica* trophozoites after 15-min incubation of FN-coated slides with live amebas and staining of the FN with anti-FN antibody and a second FITC-labeled antibody. Bar,  $25 \mu\text{m}$ . (C) Time course of the release of radioactive peptides from a  $^{125}\text{I}$ -FN substrate by *E. histolytica* (●) and *Laredo* (○) trophozoites. Each point represents the average of triplicate samples of two separate experiments.

rows) show a distinct morphology and can be seen as mouths in amebas interacting with different substrates or in trophozoites kept in suspension.

## Discussion

Fibronectin, one of the major components of the extracellular matrix, is a multifunctional glycoprotein composed of highly structured domains, one of which is a cell attachment site (14, 37). Attachment of some pathogenic organisms to fibronectin at the host cell surface has been described for bacteria (1, 9, 31) and more recently for various parasitic protozoa (26, 27, 36). Interaction with target cells has been found to require fibronectin recognition.

*Entamoeba histolytica*, the causative agent of human amebiasis, is an extracellular parasite that can produce cell lysis and extracellular matrix damage (4, 17, 25, 31). Our results show that *E. histolytica* specifically binds and degrades fibronectin. The binding is faster and more efficient than the binding showed by *Laredo* amebas, which are noninvasive but were isolated from a human carrier. *E. histolytica* trophozoites degrade the bound fibronectin, liberating smaller peptides into the medium, while *Laredo* is apparently unable to degrade the bound FN at detectable levels.

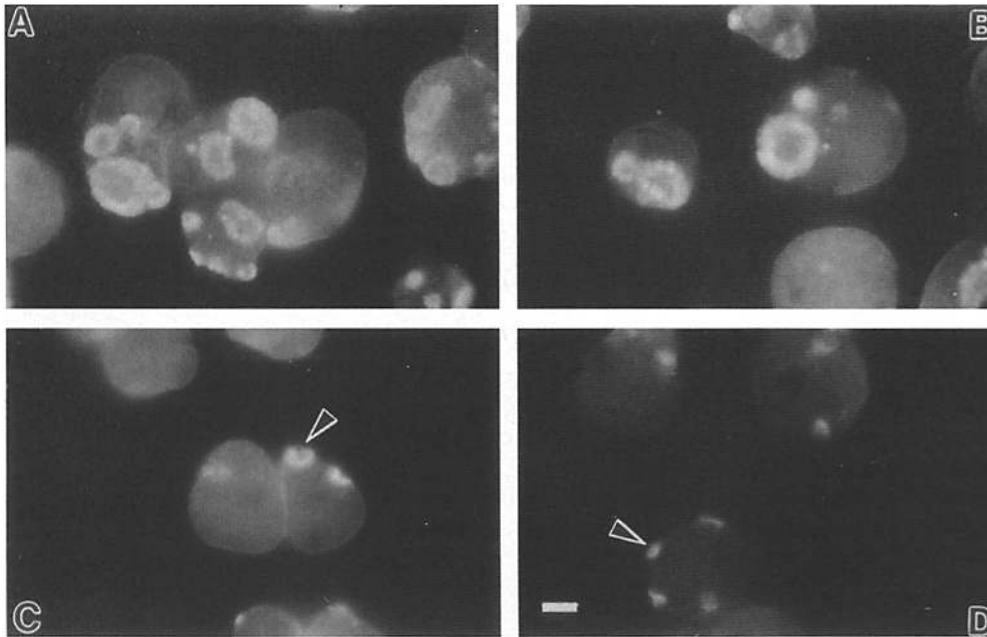
The binding was specifically inhibited by unlabeled fibronectin but not by other proteins. The difference in inhibition of binding observed between *E. histolytica* and *Laredo* was probably due to the proteolytic activities on the cell surface of *E. histolytica* trophozoites. There were also differences in the receptor affinity between the two strains because in *Laredo* saturation was not achieved even at the highest concentration of FN tested.

Because of the proteolytic activity in *E. histolytica* it was not possible to obtain data such as the number of receptor molecules/cell, the apparent dissociation constant, and the classes of receptors present in trophozoites. Protease inhibitors that effectively blocked proteolysis such as *N*-ethylmaleimide, *p*-hydroxy-mercury-benzoate, and diisopropyl-fluorophosphate could not be used with live cells as they are extremely toxic to the amebas and produce immediate lysis.

We also assayed FN to see whether it could promote trophozoite adhesion as it does in other eukaryotic cells. We found focal degradation of FN at the contact sites of the trophozoites. This could explain why the number of adhered cells decreased with time. *Laredo* on the other hand attached to FN-coated surfaces but did not degrade the protein. Localized fibronectin degradation, which has resulted in the same effects described here for *E. histolytica*, has also been reported in virus-transformed fibroblasts (34).

Release of radioactive FN peptides into the incubation medium by *E. histolytica* and not by *Laredo* further suggests that FN degradation is related to the invasive activity of pathogenic amebas. The recognition of FN and its degradation at cell contact sites may be the first step in attachment-detachment, migration, and subsequent invasion of connective tissue.

Autoradiography of thin sections of *E. histolytica* after incubation with soluble  $^{125}\text{I}$ -FN showed that part of the protein is internalized. The fact that the  $^{125}\text{I}$ -FN associated with the cell pellets and seen by SDS-PAGE was never found intact after 5–10-min interaction suggests that the internalized FN was already degraded. By contrast the  $^{125}\text{I}$ -FN associated



**Figure 6.** Actin rearrangement induced by interaction with FN. Trophozoites were allowed to interact with various substrates for 15 min, fixed, permeabilized, and stained with rhodamine phalloidin. (A and B) Amebas on fibronectin-coated glass slides. The most striking patterns of actin staining observed were the "adhesion plates." Endocytic invaginations were present (arrows) in C, amebas incubated on bovine serum albumin-coated surfaces, and (D) amebas incubated on glass. Bar, 10  $\mu$ m.

with *Laredo* trophozoites remained intact after 60 min of interaction.

Changes in the distribution of polymerized actin were elicited by the interaction between *E. histolytica* and FN. Special surface modifications, reminiscent of adhesion plates, were seen in the trophozoites only when they were interacting with FN-coated surfaces. It appears that the cytoskeletal changes at the site of FN attachment of the trophozoites were induced upon contact, presumably mediated by a receptor. They were not the consequence of or the response to contact with any protein-coated surface, as contact with BSA-coated surfaces did not elicit the formation of adhesion plates. Other eukaryotic cells (13, 29) also rearrange their cytoskeleton, particularly their actin fibers, in response to interaction with FN. *E. histolytica* trophozoites showed a response to FN apparently common to most invasive cells. It is interesting that this primitive eukaryote already has developed the capacity to respond to signals from the extracellular matrix.

A receptor-mediated mechanism would require the presence of the receptor on the amoeba's surface. We identified a single peptide of 37 kD as the putative receptor in *E. histolytica* trophozoites to which either  $^{125}$ I-FN or unlabeled FN bind in a specific manner.

In summary, we have demonstrated that FN can bind specifically to the surface of live *E. histolytica* and *Laredo* amebas. In fact, a putative receptor protein of 37 kD has been identified by FN-binding assays. *E. histolytica* degrades and internalizes the FN to which it attaches, while *Laredo* does not show this response. Presumably this proteolytic activity is an essential factor for tissue invasion. Moreover, the attachment of amebas to FN-coated surfaces appears to cause major rearrangements of their cytoskeleton, as revealed by the changes in the phalloidin staining pattern.

Further characterization of the receptor will be necessary to establish its complete identity and similarity with other FN receptors, as well as its role in the migration through and invasion of tissues by pathogenic amebas.

We acknowledge the excellent technical assistance of A. Rios, F. Cázares, and G. Rosas and thank Drs. V. Tsutsumi and C. Argüello for their valuable advice regarding autoradiography and immunofluorescence.

This research was supported by National Institutes of Health grant AI-21304, Cosnet of México, and the Rockefeller Foundation and McArthur Foundation.

Received for publication 10 September 1987.

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