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Cell-matrix interactions of *Entamoeba histolytica* and *E. dispar*. A comparative study by electron-, atomic force- and confocal microscopy

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ABSTRACT

Invasion of tissues by *Entamoeba histolytica* is a multistep process that initiates with the adhesion of the parasite to target tissues. The recognition of the non-invasive *Entamoeba dispar* as a distinct, but closely related protozoan species raised the question as to whether the lack of its pathogenic potential could be related to a weaker adhesion due to limited cytoskeleton restructuring capacity. We here compared the adhesion process of both amebas to fibronectin through scanning, transmission, atomic force, and confocal microscopy. In addition, electrophoretic and western blot assays of actin were also compared. Adhesion of *E. histolytica* to fibronectin involves a dramatic reorganization of the actin network that results in a tighter contact to and the subsequent focal degradation of the fibronectin matrix. In contrast, *E. dispar* showed no regions of focal adhesion, the cytoskeleton was poorly reorganized and there was little fibronectin degradation. In addition, atomic force microscopy using topographic, error signal and phase modes revealed clear-cut differences at the site of contact of both amebas with the substrate. In spite of the morphological and genetic similarities between *E. histolytica* and *E. dispar* the present results demonstrate striking differences in their respective cell-to-matrix adhesion processes, which may be of relevance for understanding the invasive character of *E. histolytica*.

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1. Introduction

Amebiasis, the infection of the human gastrointestinal tract by the protozoan parasite *Entamoeba histolytica,* is a significant cause of morbidity and mortality in developing countries. *E. histolytica* is capable of invading the intestinal mucosa and spreading to other organs, mainly the liver. After many years of debate, *Entamoeba dispar*, an ameba morphologically similar to *E. histolytica,* that also colonizes the human gut, was formally recognized as a separate but closely related species with no invasive potential, as originally proposed by Brumpt in 1925 [1,2]. With the availability of axenic *E. dispar* cultures [3] amebic research focused on explaining the dramatic differences in the pathogenic potential between the two species. More recently, the sequencing of the genome of both amebas has allowed whole-genome-scale analysis of genetic differences and differential gene expression to be undertaken. These studies have helped to elucidate mechanisms of virulence and identified genes differentially expressed in both species [4].

During penetration of the intestinal mucosa, *E. histolytica* adheres to the epithelium and degrades extracellular matrix (ECM) components [1,5–10], whereas *E. dispar* is not able to break this mucosal barrier and remains in the intestine without causing disease [11,12]. In vitro adhesion of *E. histolytica* trophozoites to fibronectin (FN) has been shown to occur through a specific integrin-like amebic FN receptor, which corresponds to the intermediate chain of the Gal–GalNAc amebic lectin (β1*Eh*FNR) [13]. Binding of this receptor to FN, results in the activation of different signaling pathways that affect the organization of the actin cytoskeleton [14].

The aim of this study was to comparatively analyze the adhesion process to FN of *E. histolytica* and *E. dispar* to help explaining differences in the invasive potential of the two parasites. The morphological changes occurring during the process of adhesion to FN were analyzed by diverse microscopy techniques, including scanning and transmission electron microscopy, confocal

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microscopy and atomic force microscopy. Comparative biochemical studies on the actin cytoskeleton were carried out by electrophoresis and western blot.

Differences in the organization of the actin cytoskeleton of the two species of amebas during adhesion to FN were observed. In *E. histolytica,* the plasma membrane forms focal regions of close contact with the FN substrate where actin aggregates co-localize, and extensive degradation of FN occurs. In contrast, adhesion of *E. dispar* to FN does not produce specialized contact regions, rearrangement of the actin cytoskeleton, nor degradation of FN.

2. Materials and methods

2.1. Cells

Trophozoites were cultured in borosilicate glass tubes for 72 h at 36 °C under axenic conditions in TYI-S-33 (*E. histolytica* strain HM-1:IMSS) or in YI-S (*E. dispar* strain SAW 760) medium containing 10% bovine serum and vitamins [15,16]. Parasites were harvested by chilling the culture tubes at 4 °C for 10 min, and collected after centrifugation at 900 × *g* for 5 min.

2.2. Fibronectin purification and fibronectin-coated coverslips preparation

Fresh human blood was collected in 10^{-4} M phenylmethylsulfonyl fluoride and 5% sodium citrate. FN was purified from plasma by gelatin-sepharose affinity chromatography [17]. The purified FN was dialyzed against 0.15 M NaCl, 0.05 M Tris–HCl, pH 7.4, and stored at −70 °C. Protein purity was monitored in 5% SDS-discontinuous polyacrylamide gels. Purified plasma FN molar concentration was quantified from its measured absorbance at 280 nm, where 1 mg/ml give 1.28 Optical Densities. For Fn binding and degradation assays, trophozoites were incubated on glass or FN-coated coverslips; these coverslips were prepared by adding 100 μg/ml of human plasma FN in 0.05 M Tris–HCl followed by overnight incubation of the slides under ultraviolet light at room temperature, to evaporate the buffer and to sterilize them.

2.3. Scanning electron microscopy

After 1 h incubation of trophozoites on glass or FN-coated coverslips at 37 °C, samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2, dehydrated with increasing concentrations of ethanol, critically-point dried with liquid CO_2 (31 °C and 1100 psi) using a Samdri 780 apparatus (Tousimis Corp., Rockville, MD), and coated with gold particles in an ion sputtering device (JEOL JFC-1100). Samples were then examined with a field emission JEOL-JSM 7100F scanning electron microscope (JEOL Ltd., Tokyo, Japan).

2.4. Transmission electron microscopy

After incubation for 1 h on FN-coated coverslips (100 μg/ml), samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at room temperature, postfixed with 1% (*w*/*v*) osmium tetroxide, and dehydrated with increasing concentrations of ethanol. Samples were transferred to propylene oxide, later to a mixture of propylene oxide/epoxy resin (1/1) and finally embedded in epoxy resins. Thin sections stained with uranyl acetate and lead citrate were observed in a JEOL JEM-1011 transmission electron microscope (JEOL Ltd. Tokyo, Japan).

2.5. Atomic force microscopy

Semi-thin sections of trophozoites incubated with FN were mounted on coverslips, stained with toluidine blue, and examined by atomic force microscopy (AFM) (Autoprobe CP Research ThermoMicroscope, Santa Barbara, CA, USA). Images were obtained using different tools available for analysis with the AFM, including the contact and tapping modes, and error signal images. Images were generated with the ProScan™ Software (version 1.9).

2.6. Electrophoresis and western blot analysis

Trophozoites (1×10^7) adhered to FN or glass were recovered from Petri dishes using a rubber policeman, washed with PBS and resuspended with Tris–HCl 0.05 M, NaCl 0.15 M, pH 7.2, and 2.5% triton X-100 containing protease inhibitors (3 mM N-ethylmaleimide, 3 mM iodoacetamide, 3 mM tosyl-lysine-chloromethyl-ketone, and 1 mM phenylmethylsulfonyl fluoride). the cell suspension was sonicated at 4 °C for 10 min (5 s sonication followed by 5 s on ice), in an ultrasonic cell Crusher sonic-150 W (MRC, Beijing, China). total extracts were centrifuged at 7500 × *g* for 20 min at 4 °C. the soluble fraction (sf) was separated from the pellet (if). protein concentration was determined by Bradford's method with a DC protein assay (bio-RAD, Hercules, CA, USA). Equal amounts of protein (20 μg) from both fractions were run on 10% SDS-PAGE under reducing conditions. proteins were then transferred onto nitrocellulose membranes (bio-RAD, Hercules, CA), blocked with 10% nonfat dry milk in PBS for 1 h at room temperature, washed, and incubated overnight at 4 °C with an anti-actin antibody (clone C4, EMD Millipore, Billerica, MA, USA). membranes were washed with PBS, and then incubated with a goat anti-mouse antibody conjugated to HRP (Jackson ImmunoResearch, Baltimore, MD, USA) for 2 h at room temperature. after washing with PBS, actin was detected by chemiluminescence using the SuperSignal® west Femto maximum sensitivity substrate kit according to the manufacturer's instructions (ECL, bio-RAD, Hercules, CA, USA). an anti-L220 lectin antibody was used to verify equal loading concentration of all samples (data not shown). Bands were analyzed using the ImageJ software.

2.7. Confocal microscopy

Trophozoites cultured for 72 h were chilled in an ice–water bath for 5 min, pelleted by centrifugation at 900 × *g* for 5 min, and resuspended in serum-free medium at 2.5×10^5/ml to laid on a FN-coated coverslip (100 μg/ml), and incubated for 1 h at 37 °C. Parasites were fixed with 4% paraformaldehyde for 1 h at room temperature, washed with PBS, and blocked for 1 h with 10% FBS diluted in PBS. Some samples were labeled with rhodamine–phalloidin (1:50) (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C, washed, and mounted with DAPI-Vectashield (Vector Laboratories; Ontario, Canada) onto coverslides. Other samples were permeabilized with 0.1% Triton-X100 and incubated overnight at 4 °C with an anti-FN rabbit antibody prepared in our laboratory (1:100). Cells were washed with PBS, and a FITC-labeled mouse anti-rabbit IgG (Invitrogen, Waltham, MA, USA) was used as secondary antibody and incubated for 1 h at 36 °C. A second labeling of these cells with rhodamine–phalloidin was performed as described above. After extensive washes, samples were mounted with DAPI-Vectashield onto coverslides and observed in a LSM700 Laser Scanning Microscope (Carl Zeiss Microimaging GmbH, Germany).

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