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Evidence for a novel *Entamoeba histolytica* lectin activity that recognises carbohydrates present on ovalbumin

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ABSTRACT

Entamoeba histolytica, an intestinal amoeba that causes dysentery and liver abscesses, acquires nutrients by engulfing bacteria in the colonic lumen and phagocytoses apoptotic cells during tissue invasion. In preliminary studies to identify ligands that stimulate amoebic phagocytosis, we used ovalbumin immobilized on latex particles as a potential negative control protein. Surprisingly, ovalbumin strongly stimulated *E. histolytica* particle uptake. Experiments using highly purified ovalbumin confirmed the specificity of this finding. The mechanism of particle uptake was actin-dependent, and the *Entamoeba* phagosome marker amoebapore A localised to ovalbumin-bead containing vacuoles. The most well described amoebic receptor is a Gal/GalNAc-specific lectin, but D-galactose had no effect on ovalbumin-stimulated phagocytosis. Ovalbumin has a single N-glycosylation site (Asn292) and is modified with oligomannose and hybrid-type oligosaccharides. We used both trifluoromethanesulfonic acid and N-glycanase to deglycosylate ovalbumin and tested the effect. Both methods substantially reduced the stimulatory effect of ovalbumin. Biotinylated ovalbumin bound the surface of fixed *E. histolytica* trophozoites saturably; furthermore, denatured ovalbumin and native ovalbumin both specifically inhibited ovalbumin-biotin binding, but deglycosylated ovalbumin had no effect. Collectively, these data suggest that *E. histolytica* has a previously unrecognised surface lectin activity that binds to carbohydrates on ovalbumin and stimulates phagocytosis.

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1. Introduction

Entamoeba histolytica is the intestinal protozoan parasite that causes amoebiasis, a disease characterised by dysentery and liver abscesses (Haque et al., 2003). Phagocytosis of host erythrocytes and immune cells is a prominent pathological feature of invasive amoebiasis, and *E. histolytica* mutant strains defective in phagocytosis have reduced virulence in animal models of disease (Griffin, 1972; Orozco et al., 1983; Rodriguez and Orozco, 1986). Phagocytosis of bacteria, furthermore, serves as an essential source of nutrition for amoebic trophozoites in the colonic lumen (Rees et al., 1941; Jacobs, 1947; Nakamura, 1953).

Entamoeba histolytica induces host cell apoptosis using a contact-dependent mechanism, and it phagocytoses apoptotic cells more efficiently than healthy cells (Huston et al., 2000, 2003). At least two features present on the surface of apoptotic cells stimulate *E. histolytica* phagocytosis: phosphatidylserine, which is exposed during apoptosis and following calcium ionophore-treatment of erythrocytes, and the human serum protein C1q, which becomes concentrated on apoptotic blebs (Huston et al., 2003; Boettner et al., 2005; Teixeira et al., 2008). Induction of host cell apoptosis requires adherence that is mediated by a galactose/N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin on the amoebic surface (Petri et al., 1987; Huston et al., 2000). However, D-galactose at concentrations that almost completely inhibit amoebic adherence to and killing of host cells only inhibits phagocytosis of already apoptotic cells by approximately 40%, indicating the existence of phagocytosis receptors in addition to the Gal/GalNAc-specific lectin (Huston et al., 2003). Additional amoebic surface proteins that have been implicated in phagocytosis of host cells include a glycosylphosphatidylinositol (GPI)-anchored serine-rich *E. histolytica* protein (the SREHP), a 112 kDa adhesin that is comprised of two proteins and possesses proteinase activity, and a recently identified phagosome-associated transmembrane kinase (Garcia-Rivera et al., 1999; Boettner et al., 2008; Teixeira and Huston, 2008).

Here, we present data indicating that *E. histolytica* has at least one previously unrecognised surface lectin activity that facilitates phagocytosis. We attempted to use hen egg white

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albumin (ovalbumin; Ova) as a negative control protein in preliminary studies using protein-coated latex beads as targets for *E. histolytica* phagocytosis, and were surprised to find that Ova is a strong and specific stimulant of *E. histolytica* phagocytosis. Of course, there is no reason to believe that *E. histolytica* interacts with Ova in the human colon. However, Ova has a single N-glycosylation site (Asn_{292}) with a heterogeneous mixture of attached neutral glycans, mostly of the unprocessed high-mannose type or partially processed hybrid variety ($Man_5GlcNAc_2$, $Man_5GlcNAc_3$, $Man_5GlcNAc_4$, and $Man_6GlcNAc_2$ collectively account for more than 60%) (Nisbet et al., 1981; Thaysen-Andersen et al., 2009). Given this and the prevalence of lectin–carbohydrate pairings that mediate host–microbe and microbe–microbe interactions, we hypothesised that *E. histolytica* has a surface lectin activity that facilitates phagocytosis and serendipitously recognises carbohydrates on Ova. Consistent with this, we demonstrated that deglycosylation of Ova eliminates its ability to stimulate amoebic phagocytosis, and that labelled Ova shows saturable and specific binding to the surface of amoebic trophozoites.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were purchased from Fisher Scientific (USA) unless otherwise noted. Ova (90% pure, Grade III), BSA (fraction V), D-mannose, D-maltose, D-fucose, D-lactose, D-xylose, heparin sulphate, chondroitin sulphate, porcine stomach mucin, N-acetylglucosamine, cytochalasin D and anhydrous trifluoromethanesulfonic acid (TFMS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). N-Glycanase (PNGaseF) was purchased from New England BioLabs (Ipswich, MA, USA). N-Acetylgalactosamine (GalNAc)–BSA was obtained from Accurate Chemicals (Westbury, NY, USA), and Endo-Grade Ovalbumin (>98% pure; endotoxin concentration <1 EU/mg) (E-Ova) was purchased from Profos AG (Regensburg, Germany). Sulfo-NHS-LC-biotin was purchased from Pierce Biotechnology (Rockford, IL, USA). Streptavidin-Alexa Fluor 488 and the goat anti-rabbit IgG Alexa Fluor 633 antibody were purchased from Invitrogen (Carlsbad, CA, USA), and the anti-amoebapore A polyclonal rabbit IgG was a gift from Matthias Leippe (Christian-Albrechts-Universität zu Kiel, Kiel, Germany).

2.2. Cultivation of E. histolytica

Entamoeba histolytica trophozoites (strain HM-1:IMSS) were grown axenically in trypticase-yeast extract-iron serum (TYI-S-33) medium supplemented with 100 μg/ml streptomycin sulphate and 100 U/ml penicillin at 37 °C as previously described (Diamond et al., 1978). Trophozoites were used during log-phase growth and harvested for experiments by incubation on ice for 10 min, centrifugation at 200 × g, washing twice with PBS, and resuspended in PBS.

2.3. Preparation of single-ligand fluorescent particles

Fluorescent latex particles coated with a single protein were used to examine the ability of potential protein ligands to stimulate *E. histolytica* phagocytosis (Teixeira et al., 2008). For this, proteins were biotinylated using Sulfo-NHS-LC-biotin, dialysed against PBS, and bound to streptavidin-coated 2 μm fluorescent latex particles (Polysciences Inc. (Warrington, PA, USA)). The biotin-labelling reactions were conducted according to the manufacturer's protocol. Streptavidin-coated latex particles were then washed four times with PBS containing 1% BSA (PBS/BSA), resuspended in PBS/BSA at a concentration of 5×10^8 beads/ml, and incubated with 20 μg/ml of biotinylated protein for 30 min at 4 °C. Coated particles were washed three more times with PBS/BSA prior to use.

2.4. Deglycosylation of protein ligands

Chemical deglycosylation was performed with TFMS according to the method of Edge et al. (1981). Briefly, 1 mg of endotoxin-free Ova was placed in a dry glass reaction vial and capped with a Teflon-faced seal (Thermo Scientific). Sixty microlitres of toluene was added to a glass ampoule containing 540 μl of anhydrous TFMS using a dry glass syringe (Hamilton) and stainless steel needle. The protein sample was cooled using a dry ice/ethanol bath for approximately 20 s, 50 μl of the TFMS/toluene mixture was added and the vial was placed in the freezer (−20 °C). It was agitated at 5 and 10 min, and then incubated for 4 h at −20 °C. After incubation, the vial was placed back on dry ice/ethanol and a mixture of pyridine/methanol/water (3:1:1 by volume) was added slowly. The vial was transferred to dry ice for 5 min and then wet ice for an additional 15 min, after which the reaction was stopped by addition of 400 μl of 0.5% [w/v] ammonium bicarbonate. The sample was then centrifuged (16,000 × g, 15 min, room temperature), the supernatant was removed and discarded, and the protein pellet was resuspended in PBS. Enzymatic deglycosylation was performed using PNGaseF according to the manufacturer's instructions. For this, 600 μg of E-Ova was denatured in 0.5% SDS and 40 mM DTT for 10 min at 100 °C. PNGaseF (3500 units) was added and a manufacturer supplied reaction buffer was added to achieve a final concentration of 50 mM sodium phosphate (pH 7.5) and 1% NP-40. The mixture was incubated at 37 °C for 1 h. Protein integrity and deglycosylation were assessed as in Section 2.5.

2.5. Protein analysis

Where indicated, protein ligands to be immobilized on single-ligand particles were analysed for purity, integrity, success of deglycosylation and success of biotinylation. Protein ligands were resuspended in Laemmli sample buffer, boiled for 5 min and separated by SDS–PAGE on 12% polyacrylamide gels (Laemmli, 1970). The effectiveness of protein deglycosylation was determined by staining gels with Periodic Acid Schiff (PAS) stain (GelCode Glycoprotein Staining Kit, Pierce Biotechnology) according to the manufacturer's instructions. This method uses periodic acid to oxidise glycols present on glycoproteins to aldehydes, which are then revealed by treatment with a reducing reagent. After PAS staining, the purity and integrity of non-deglycosylated and deglycosylated proteins were assessed by silver staining the same gel to visualise all proteins (Rabilloud, 1992). Successful biotinylation of proteins to be bound to streptavidin-coated latex particles was confirmed by immunoblotting. Following biotinylation using Sulfo-NHS-LC-biotin, equal quantities of the protein ligands were separated as above by SDS–PAGE on 12% polyacrylamide gels, transferred to polyvinyl difluoride (PVDF) membranes (Millipore Corp.), blocked for 1 h at room temperature with Tris–buffered saline–1% Tween 20 (pH 7.4) containing 5% BSA, and probed overnight with a streptavidin–horseradish peroxidase (HRP) conjugate (Amersham Pharmacia). Bound streptavidin–HRP was visualised using enhanced chemiluminescence (Amersham Pharmacia).

2.6. Phagocytosis assays

Unless otherwise noted, phagocytosis experiments were conducted in the presence of D-galactose to inhibit the amoebic Gal/GalNAc-specific surface lectin. The flow cytometry phagocytosis assay has been described elsewhere (Huston et al., 2003). Briefly,

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