Infection and Immunity Mediated by the Carbohydrate Recognition Domain of the Entamoeba histolytica Gal/GalNAc Lectin

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Entamoeba histolytica causes invasive amebiasis, a major parasitic disease of the developing world, whose primary symptoms are liver abscess and colitis. All strains of E. histolytica express a 260-kDa surface Gal/GalNAc lectin that is antigenically conserved and immunogenic. The lectin is required for adherence to human intestinal epithelial cells and contact-dependent killing of immune effector cells. By expression cloning, the carbohydrate recognition domain (CRD) was identified within the lectin heavy-subunit cysteine-rich region. Of interest for a hepatic parasite, the CRD had sequence identity to the receptor-binding domain of hepatocyte growth factor (HGF) and competed with HGF for binding to the c-Met HGF receptor. In an animal model of invasive disease, immunization with the CRD inhibited liver-abscess formation, yet in humans, a naturally acquired immune response against the CRD did not persist.

The World Health Organization estimates that annually 50 million cases of colitis and liver abscess and 100,000 deaths result from Entamoeba histolytica infection [1]. Most of the infections occur in the developing nations of Central and South America, Africa, and Asia. Microbial adhesion is often a first step during infections leading to overt disease. E. histolytica adheres to and destroys cells in the human gut, causing severe tissue damage in the colon and sometimes abscesses in the liver. Therapies aimed at blocking adhesion may prevent colonization and subsequent disease. Since humans are the only reservoir for E. histolytica, a vaccine that prevents colonization by blocking adherence could eradicate amebiasis.

E. histolytica colonizes and invades tissues via a surface adhesin-lectin that binds exposed terminal galactose/N-acetyl-D-galactosamine (Gal/GalNAc) residues of target cell glycoproteins or glycolipids. Inhibition of the Gal/GalNAc lectin prevents amebic killing of host cells [2]. The purified 260-kDa E. histolytica lectin dissociates under reducing conditions into heavy (170 kDa) and light (31–35 kDa) subunits [3]. The heavy subunit contains a putative transmembrane domain, and the light subunit is glycosylphosphatidylinositol (GPI) anchored [4], an unusual combination. The lectin is an obvious vaccine candidate. However, animals immunized with the intact lectin produce antibodies against adherence-enhancing and -inhibiting epitopes on the lectin heavy subunit, and in some cases, disease is exacerbated [5]. Better results are obtained using recombinant subunit vaccines that protect animals from liver-abscess formation without disease enhancement [6–8].

Humans remain infected with E. histolytica despite an anti-lectin antibody response, which also includes adherence-enhancing and -inhibiting anti-lectin antibodies [9, 10]. Our goal was to define the carbohydrate recognition domain (CRD), because an immune response against it should be more predictably adherence-inhibitory and protective.

We hypothesized that the lectin heavy subunit contained the CRD. The evidence to support this was that anti–heavy-subunit monoclonal antibodies (MAbs) inhibit or enhance the carbohydrate-binding activity of the lectin [9, 10], while anti–light-subunit MAbs do not [4]. Sequence analysis has not identified a CRD in either subunit. This is not surprising, because in other Gal/GalNAc-binding lectins only the spatial relationship between the plane formed by the C3–C6 carbons of the Gal residues and an aromatic side chain (tryptophan, tyrosine, or phenylalanine) in the binding site is conserved [11].

The epitope of the adherence-inhibitory MAb 8C12 maps by deletion analysis [9] to a 104-aa portion of the lectin heavy subunit (aa 895–998, CRD; figure 1A). The sequence of the 104-aa fragment is completely conserved among other members of the E. histolytica lectin gene family, and 89% conserved in the homologous protein expressed by the closely related non-invasive parasite Entamoeba dispar [12]. Here we report the
Figure 1. Identification of carbohydrate recognition domain (CRD). A. Putative structural domains of lectin heavy subunit. Epitope recognized by adherence-inhibitory monoclonal antibody (MAb) 8C12 is located between aa 895 and 998 (denoted CRD). CW, cysteine-tryptophan domain; CF, cysteine-free domain; TM, transmembrane domain. B. CRD and CW domains expressed in *Escherichia coli* as polyhistidine fusion proteins and purified via nickel affinity chromatography. Fusion proteins were analyzed by SDS-PAGE followed by (1) Coomassie blue stain, (2) Western blot with MAb 8C12, and (3) Western blot with 125I-labeled synthetic multivalent neoglycoprotein GalNAc20BSA (mean of 20 GalNAc residues linked to bovine serum albumin).

Identification of a CRD within this 104-aa fragment of the *E. histolytica* lectin, and explore the role of an antibody response against the CRD in immunity to amebiasis.

**Materials and Methods**

*Production and purification of polyhistidine fusion proteins.* The cysteine-tryptophan–rich (CW) and CRD fragments were amplified by polymerase chain reaction of *E. histolytica* genomic DNA using primers containing restriction sites (forward primers contained a BamHI site; reverse primers contained an EcoRI site) to facilitate cloning into a pRSET-A vector (Invitrogen, Carlsbad, CA): CW forward, CGCGGATCCGATAAACTTGATGAATT; CW reverse, CGCGGATCCACATTTTGATGTAGTGTTTG; CRD forward, CGCGGATCCGCATATTGTACATACGA; CRD reverse, CGCGGATCCGCATTTTGATGTAGTGTTTG; CRD reverse, CGCGGATCCGCATTTTGATGTAGTGTTTG. The sample was concentrated using an ultrafiltration unit (CentriPlus-50; Millipore, Bedford, MA).

Preparative cell electrophoresis was done using a 491 Prep Cell apparatus (BioRad, Hercules, CA) with a 12%, 9.5-cm (long) resolving gel for CRD and a 10% 9.5-cm resolving gel for CW. The stacking gel was 4%, 2.5 cm, for both proteins. Gels were made from a 30% acrylamide stock solution (37.5:1 acrylamide:bis-acrylamide). Running buffer was 25 mM Tris base, 190 mM glycine, 0.1% SDS. Concentrated protein samples were layered on the gel and electrophoresed at 12 W constant power. Fractions were collected after the loading dye passed through the elution chamber.

Western blots. Two micrograms of each purified polyhistidine CW or CRD fusion protein was electrophoresed in SDS-polyacrylamide (12%) gels and then transferred to a polyvinylidine difluoride (PVDF) membrane at 100 V for 1 h using a Mini TransBlot apparatus (BioRad) per the manufacturer’s instructions. For Western blots with MAb 8C12, the membrane was blocked for 1 h at 4°C in 5% nonfat dry milk in blot wash buffer (50 mM NaCl, and 0.1% Tween 20, at 7.4 pH), then probed with 20 μg/mL MAb 8C12 in 1% nonfat milk–blot wash buffer for 1 h at 4°C. For Western blot with 125I-GalNAc200 bovine serum albumin (BSA), 2 μg of each polyhistidine CW or CRD fusion protein was run on an SDS-polyacrylamide (12%) gel and then transferred to PVDF. The blot was air dried at room temperature, rewet in methanol, and blocked in 1% BSA in PBS, then incubated with 2 × 10^6 cpm/mL 125I-GalNAc200 BSA [13] in 0.1% BSA/PBS for 1 h at room temperature. The blot was washed 3 times for 5 min in 0.1% BSA/PBS, air dried, then visualized with a phosphor imager. For dot blots probed with 125I-GalNAc200 BSA, 1 μg of immunoadsorption–purified lectin [3] and 2 μg of his-CW or his-CRD were applied to PVDF membranes using a vacuum manifold (Bio-Dot; BioRad). Blots were processed as above with the addition of increasing amounts of asialofetuin or fetuin during the 125I-GalNAc200 BSA incubation step. Blots were washed 3 times for 5 min in 0.1% BSA/PBS, air dried, and visualized with a phosphor imager. Quantitation was done using ImageQuant (Molecular Dynamics, Sunnyvale, CA) software with local median background correction. For calcium-dependence experiments, the dot blots were blocked in 1% BSA in 10 mM HEPES, at 7.4 pH, then probed with 2 × 10^6 cpm/mL 125I-GalNAc200 BSA in 0.1% BSA/HEPES plus increasing...
Inhibition of hepatocyte growth factor (HGF) binding. The binding of recombinant HGF to a c-Met receptor IgG fusion protein was measured using a modification of the microtiter plate assay previously described [14].

Immunization studies. Male Mongolian gerbils were immunized with purified his-CRD. The his-CRD was dialyzed against PBS to partially remove SDS and β-mercaptoethanol. This produced a partially particulate mixture that was emulsified in complete Freund's adjuvant. Gerbils were immunized intraperitoneally with 200 µg of his-CRD in complete Freund's adjuvant. Animals were boosted at 2 weeks with 200 µg and at 4 weeks with 400 µg of his-CRD in incomplete Freund's adjuvant. Control gerbils were injected with complete Freund's adjuvant and at weeks 2 and 4 with incomplete Freund's adjuvant alone. About 0.8 mL of blood was collected by retro-orbital puncture from each animal after the last boost. Gerbils were challenged intrahepatically at 6 weeks with 10⁶ amebas; animals were sacrificed 10 days after challenge, and liver-abscess size was measured. For the passive immunization trial, gerbils received 0.1 mL of sera from control or CRD-immunized animals intramuscularly 24 h before challenge, followed by an additional 0.1 mL of sera intrahepatically at the time of challenge.

Determination of human serum antibody levels. To study the persistence of anti-CRD antibodies, serum samples from patients in South Africa with amebic liver abscess were analyzed. Serum antibody levels against the lectin, the CRD, or the CW domain were determined by ELISA using 96-well plates coated with 0.25...
Results

Identification of the CRD domain. The CRD and the unrelated amino-terminal CW domain of the lectin were expressed in E. coli as recombinant polyhistidine fusion proteins. As expected, MAb 8C12 recognized the purified CRD fusion protein (figure 1B, panel 2). Two adherence-enhancing MAbs (3F4 and 8A3) that mapped to the CRD by deletion analysis [9] did not recognize the polyhistidine CRD fusion protein on a Western blot (data not shown). This suggests that these MAbs recognized conformational epitopes or epitopes located elsewhere on the protein that depended on the presence of the CRD for their structures. Two other adherence inhibitory MAbs recognized epitopes on either side of the 104-aa fragment, each ~100 aa away [9]. Therefore, epitope mapping pointed to, but did not unambiguously identify, the region from aa 895–998 as the CRD of the E. histolytica lectin.

We used a GalNAc-containing neoglycoprotein (a synthetic glycoprotein) to test the hypothesis that the 104-aa CRD fragment contained a CRD. E. histolytica membranes bind to the polyvalent neoglycoprotein, GalNAc₂₀BSA, with 500,000-fold higher affinity than to GalNAc monosaccharide [13]. The purified CRD polyhistidine fusion protein bound ¹²⁵I-labeled GalNAc₂₀BSA (2 × 10⁵/mL) (figure 1B, panel 3), while the CW protein did not. Binding of GalNAc₂₀BSA by the CRD fusion protein was specifically inhibited by the Gal/GalNAc-terminal glycoprotein asialofetuin (figure 2A). The slight inhibition of binding by 1 μM fetuin was probably due to trace amounts of desialylated glycoprotein commonly found in commercial preparations.

Calcium dependence of ligand binding by the CRD. Binding of ¹²⁵I-labeled mucin glycoproteins by E. histolytica trophozoites is calcium dependent [15], and Gal/GalNAc binding by E. histolytica membranes (implicating the lectin) is Ca²⁺ dependent at low ionic strength [13]. Intraluminal sodium ion concentration in the colon is ~30 mM [16]. As expected, both the purified lectin and the CRD bound ligand in a calcium-dependent manner (figure 2B). Calcium ions can stabilize or alter protein structures [17–19] or provide a coordinating bridge between lectins and their ligands as in the case of mannose-binding protein and other C-type lectins [20–22]. By binding Ca²⁺, the CRD may adopt a more favorable conformation for the ligand or the CRD may bind Ca²⁺ so as to allow interactions between it and the hydroxyl oxygens of the ligand. In either case, the ability of the lectin to bind calcium ions may be crucial for its function in the colon.

CRD inhibition of HGF binding to c-Met. Sequence analysis of the CRD revealed it had limited sequence identity to the
receptor-binding domain of HGF (figure 3A) [23]. Therefore, we tested the ability of the CRD to compete with HGF for binding to its receptor, c-Met. The binding of recombinant HGF to a c-Met–IgG fusion protein was measured by a microtiter plate assay [14]. HGF binding to c-Met competed with excess CRD or native lectin but not with the unrelated CW fusion protein (figure 3B). The competition of HGF binding to c-Met was not due to the carbohydrate-binding activity of CRD: Inhibition of HGF binding to c-Met by 2 μM his-CRD was 54% ± 5.1% with and 56% ± 2.6% without 50 mM GalNAc.

**Immunization with the CRD.** A 375-aa portion of the lectin, which contains the CRD, protects gerbils from liver-abscess formation and produces an intestinal secretory IgA response that inhibits adherence [7, 24]. Others have seen antibody-mediated protection of gerbils immunized with a fragment of the lectin, which contains 60 carboxyl residues of the CRD [25]. In light of these results and our demonstration of carbohydrate-binding activity of the CRD, we immunized gerbils with the CRD fusion protein and tested the antiserum (1:5 dilution) for inhibition of amebic adherence [2] to target cells. The anti-CRD antiserum almost completely inhibited adherence (figure 4). No enhancement of adherence by the anti-CRD antiserum was observed at any dilution, unlike previous results with gerbil anti-lectin antiserum.

After direct intrahepatic challenge of the gerbils with *E. histolytica* trophozoites, animals that were actively immunized with the CRD developed a mean liver-abscess weight that was 18% of that in sham-immunized control animals (P < .05, table 1). Importantly, the exacerbation of disease seen in a subset of animals immunized with the native lectin [5] was not observed in CRD-immunized animals. Other investigators have observed similar levels of protection in animals immunized with portions of the cysteine-rich domain that encompass the CRD [6–8, 25]. We therefore tested whether the protection that we observed with CRD immunization was antibody mediated. Passive transfer of sera from CRD-immunized gerbils to naive animals at the time of challenge conferred protection at a level equivalent to the active immunization (table 1).

**Anti-CRD antibody response in humans.** Anti-CRD, anti-lectin, and anti-CW serum antibody levels of patients from South Africa with current or past *E. histolytica* infection were measured. It was previously demonstrated that antibody levels against the lectin cysteine-rich domain (which includes the CRD) decline with time after infection [26]. The antibody response against the entire lectin was vigorous (>1.0 absorbance at 450 nM) in all patients with current infections. Recognition by serum antibodies of the entire lectin also persisted in patients with previous infection. In comparison, many currently infected patients had a strong response against the CRD, but it was barely detected in people with past infections (figure 5). Antibody responses against the CW domain were also strong in people with current *E. histolytica* infections and were present at a diminished level in those with past infections.

## Discussion

Our major findings were the identification of the CRD of the *E. histolytica* Gal/GalNAc adherence lectin and demonstration that an adherence-inhibitory antibody response against this domain protects against amebic liver abscess in an animal model. In addition, we showed that the CRD (which has sequence identity to the receptor-binding domain of HGF) competed with HGF for binding to the c-Met receptor. Binding of the Gal/GalNAc lectin (via its CRD) to c-Met on hepatocytes might explain the liver tropism of *E. histolytica*.

The identification of a CRD in the Gal/GalNAc lectin is an important step toward the development of a vaccine for amebiasis. The lectin is an attractive vaccine candidate. It mediates adherence to colonic mucosa and host cells and initiates contact-dependent cytolysis. The lectin, by inhibiting assembly of the complement membrane attack complex, also plays a role in parasite serum resistance [27]. Inhibition of wild-type lectin function by inducible expression of dominant negative lectin mutants decreases adherence, cytolysis, and liver-abscess formation [28]. Anti-lectin antibodies can neutralize adherence, cytotoxicity, and serum resistance. Of importance, these neutralizing antibody epitopes have been antigenically conserved in all *E. histolytica* isolates examined to date [29, 30]. A potential problem, however, with the use of the lectin as a vaccine has been the presence of adherence-enhancing antibody epitopes on the heavy subunit. The CRD lacks these epitopes, and immunization with the CRD did not result in the production of adherence-enhancing antibody responses.

The precise roles and importance of humoral and cellular responses in immunity to amebiasis remain to be determined. Support for a role for antibodies in immunization-mediated protection has come not only from this work but also from studies using a severe combined immunodeficient (SCID) mouse model. Passive transfer to SCID mice of antibodies

<table>
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<th>Trial</th>
<th>Liver abscess weight (g)</th>
<th>% of control</th>
<th>P</th>
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<tr>
<td>Control</td>
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<tr>
<td>Immune</td>
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<tr>
<td>Control (sera from controls)</td>
<td>4.6 ± 1.5 (n = 4)</td>
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<tr>
<td>Immune (sera from immunized)</td>
<td>0.65 ± 1.0 (n = 4)</td>
<td>14 &lt; .05</td>
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**NOTE.** Gerbils were actively immunized with 200 μg of carbohydrate recognition domain (CRD) in complete Freund’s adjuvant and boosted with 200 μg of CRD in incomplete Freund’s adjuvant at 2 weeks and 400 μg at 4 weeks. Animals were challenged with 500,000 trophozoites at week 6. Passive immunization consisted of transfer to naive animals of 0.1 mL of control or immune sera intramuscularly 24 h before challenge and an additional 0.1 mL intrahepatically at time of challenge. Animals were sacrificed 10 days after challenge and liver-abscess weights measured.
against whole *E. histolytica* proteins, the serine-rich protein, or the cysteine-rich domain of the galactose lectin, resulted in faster resolution of amebic liver abscess [25, 31].

The lack of severe amebiasis in persons with AIDS suggests a less than stringent requirement of CD4 cells for protective immunity. However, lymphocytes from persons who have recovered from invasive amebic disease proliferate in response to amebic antigens, have amebicidal activity, and produce interleukin-2 and interferon (IFN)-γ [32]. Macrophages and neutrophils, activated with IFN-γ and tumor necrosis factor (TNF)-α, can kill *E. histolytica* trophozoites; in the absence of IFN-γ, these effector cells are killed by amebas [32, 33]. In murine macrophages, TNF-α plays a central role in activating macrophages for nitric oxide–dependent cytotoxicity against *E. histolytica* [34, 35]. While our data are consistent with anti-lectin antibodies protecting against an intrahepatic challenge, it seems likely that an effective vaccine will require induction of both cell-mediated and humoral immune responses.

It is not known if there is acquired immunity to *E. histolytica* infection in humans. In longitudinal studies of persons asymptomatically infected with *E. histolytica* (all with serum anti-amebic antibodies), most spontaneously cleared the infection in 3–9 months. However, 10%–14% of *E. histolytica*-infected persons progress to invasive amebiasis, demonstrating that immunity, if it exists, is incomplete [36–38]. It is of interest to speculate that anti-CRD antibodies may play an important role in preventing invasive infection with *E. histolytica* in humans. The need to raise and sustain a high level of anti-CRD antibodies may be an important consideration in vaccine design.
Acknowledgments

We thank R. Schwall and L. Chang for assistance with the HGF binding assays, Shirley Epstein for serum collection, and R. Schnaar, Y. C. Lee, and R. Lee for advice and discussion.

References