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MINIREVIEW

Adherence and Cytotoxicity of *Entamoeba histolytica* or How Lectins Let Parasites Stick Around

JAMES J. McCOY,[1]† BARBARA J. MANN,[2] AND WILLIAM A. PETRI, JR.[1,2,3]*

Departments of Medicine,[2] Microbiology,[1] and Pathology,[3] University of Virginia School of Medicine, Charlottesville, Virginia 22908

INTRODUCTION

The enteric protozoan parasite *Entamoeba histolytica* is one of the most potent cytotoxic cells known, named by Schaudinn in 1903 for its ability to destroy human tissues (53). Infection occurs when the cyst form of the parasite is ingested with contaminated food or water. After excysting to form the trophozoite in the small intestine, the amebae can colonize the bowel lumen, invade through the intestinal epithelium to cause colitis or liver abscess, or form cysts that are excreted with the stool to start a new round of infection (28).

Parasite recognition of glycoconjugates plays an important role in the pathogenesis of amebiasis. Killing of host cells by *E. histolytica* trophozoites in vitro occurs only upon direct contact, which is mediated by an amebic adhesin which recognizes N- and O-linked oligosaccharides (9, 35, 36, 49). This adhesin is specifically inhibited by millimolar concentrations of galactose and *N*-acetyl-D-galactosamine (Gal/GalNAc) (36). Avoidance of lysis by the complement membrane attack complex is also mediated by this lectin. Here we discuss recent advances in the understanding of this novel amebic adhesin.

ADHERENCE IS BLOCKED BY Gal AND GalNAc

The essential role of the Gal/GalNAc lectin in amebic adherence to target cells has been demonstrated with carbohydrate inhibitors and with target cells deficient in glycosylation. In in vitro adherence assays, the binding of *E. histolytica* trophozoites to Chinese hamster ovary (CHO) cells was inhibited 90 to 95% by 50 mM Gal and GalNAc while other sugars had no effect (4, 6, 36, 37, 50, 51). Significantly, trophozoites also exhibited Gal/GalNAc-inhibitable adherence to human immune effector cells, including neutrophils and macrophages, as well as to human colonic mucus glycoproteins and colonic epithelial cells, the relevant physiological targets of *E. histolytica* infection (6, 37). CHO cell mutants defective in the production of N- and O-linked Gal-terminal oligosaccharides were almost completely resistant to adherence. Adherence was enhanced upon removal of terminal sialic acid residues with neuraminidase, or to CHO cell mutants lacking terminal sialic acid residues, suggesting a preference for terminal Gal residues over those positioned internally (43). Complex branched Gal-terminal carbohydrates were 1,000-fold more effective by weight than Gal in inhibiting adherence to CHO cells (31). This increased affinity of lectins for branched oligosaccharides is postulated to be due to multivalent receptor-ligand interactions and has been termed the glycoside clustering effect (13).

CYTOTOXICITY IS CONTACT DEPENDENT AND EXTRACELLULAR

Adherence of *E. histolytica* trophozoites to colonic epithelium is observed prior to epithelial cell lysis and amebic invasion of the colon in animal models (5, 54). In vitro, death of CHO cells was seen by cinemicroscopy within 5 min of contact with amebae, while CHO cells not in direct contact with amebae remained viable (35). Killing was maximal at 37°C and did not occur at 4°C (36). Incubation of *E. histolytica* trophozoites in suspension with CHO cells also resulted in killing of the CHO cells, as analyzed by release of ^{111}InO or by trypan blue uptake (35). The requirement for adhesion was demonstrated in this system by suspending the cells in 10% dextran, which blocked adherence and killing (36). Contact-dependent extracellular killing of human macrophages, neutrophils, and erythrocytes and a human liver cell line by the amebae has also been observed (9, 51).

INHIBITION OF ADHERENCE VIA THE Gal/GalNAc LECTIN BLOCKS CYTOTOXICITY

Adherence and subsequent contact-dependent killing of CHO cells and other target cells is nearly completely inhibited by Gal and GalNAc but not other monosaccharides (36). Gal or GalNAc blocks killing of target cells even when trophozoites and CHO cells are brought into contact by centrifugation, indicating that engagement of the lectin with target cell glycoconjugates is required for both adherence and killing (36). Further supporting the requirement for Gal-dependent adherence in cytolysis was the finding that CHO cell glycosylation-deficient mutants lacking terminal Gal residues on N- and O-linked sugars were totally resistant to amebic adherence and cytolytic activity, whereas CHO cell mutants with increased terminal Gal residues had enhanced susceptibility to adherence and lysis (42). The separate steps of adherence and adherence-dependent cytotoxicity are depicted in Fig. 1.

A dramatic example of the importance of amebic adherence via the Gal/GalNAc lectin for host cell killing is the interaction with human neutrophils. Virulent *E. histolytica* has the ability to lyse neutrophils: at a ratio of 100 neutrophils per amebae 93% of the neutrophils were killed after 22 h of incubation at 37°C (9). Blockade of the lectin with 50 mM GalNAc not only prevented killing of neutrophils but enabled the neutrophils to destroy the amebae.

* Corresponding author. Mailing address: Room 2115 MR4 Building, University of Virginia Health Sciences Center, Charlottesville, VA 22908. Phone: (804) 924-0075. Fax: (804) 924-2885. Electronic mail address: wap3g@galen.med.virginia.edu.

† Present address: Department of Medicine, University of Iowa, Iowa City, IA 52242.

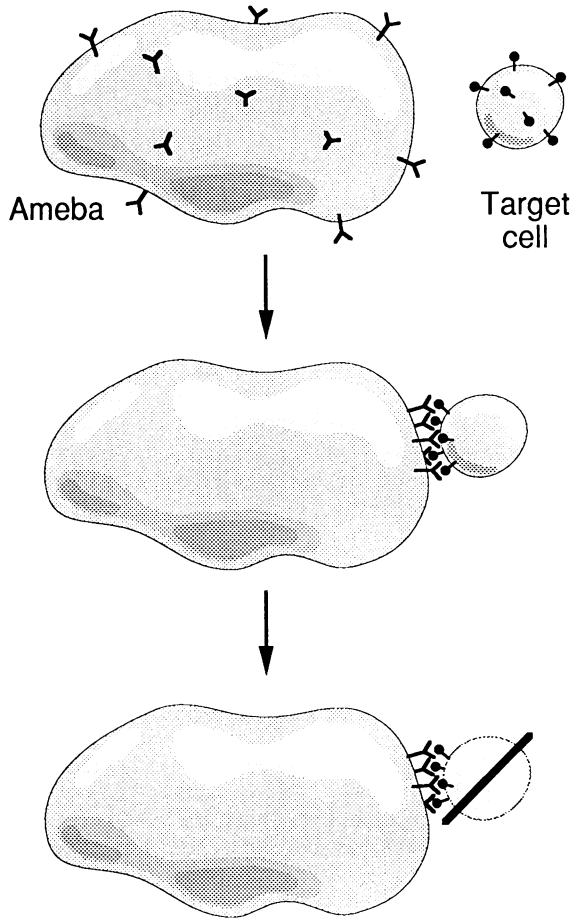

FIG. 1. Model of adherence-dependent killing. Amebae adhere to Gal/GalNAc-containing oligosaccharides (●) on target cells via a cell surface lectin (Y). Capping of lectin and target cell glycoconjugates is followed by extracellular lysis of the target cell. Engagement of the lectin with target cell glycoconjugates is required for target cell killing; artificial apposition of amebic and target cell membranes does not result in target cell killing if the lectin is blocked with Gal or GalNAc.

THE MECHANISM OF EXTRACELLULAR KILLING IS UNKNOWN

While a number of factors may be involved in contact-dependent killing by *E. histolytica*, none has been rigorously proved experimentally to be responsible for amebic cytolysis. Studies with the calcium-binding fluorescent dye Fura-2 revealed an approximately 20-fold increase in the concentration of intracellular calcium in target cells within seconds of direct contact by an amebic trophozoite followed by alterations in target cell plasma membrane permeability, as indicated by uptake of trypan blue or loss of Fura-2 dye (38). Actual cell death occurred 5 to 15 min after the lethal hit was delivered. Supporting the requirement for calcium was the finding that the calcium inhibitors EDTA and TMB-8 (40) and the slow sodium-calcium channel blockers verapamil and bepridil (41) significantly reduced amebic killing of target CHO cells in suspension. The rise in target cell intracellular calcium levels and subsequent killing by *E. histolytica* trophozoites was inhibited by Gal (38).

Isolation of amebic pore-forming proteins similar in function to pore-forming proteins of the immune system has been reported by a number of laboratories. A purified 30-kDa amebic protein was purified and demonstrated to lyse erythrocytes and to insert into and create pores in lipid bilayers (59, 60). A distinct 14-kDa amebic pore-forming protein, designated amoebapore, was described by a second group as an ion channel-forming protein (18, 46). A 5-kDa amoebapore has also been reported, and this is the only purified protein with pore-forming characteristics whose amino acid sequence has been determined. Analysis of the deduced amino acid sequence of the 5-kDa amoebapore predicts that it folds into four adjacent alpha helices and has amphiphilic characteristics similar to those of other membrane-disrupting proteins (15). Both *E. histolytica* and *Entamoeba dispar* had pore-forming activity, but the activity of the nonpathogenic *E. dispar* was 80% less than that of the pathogenic *E. histolytica*. In these experiments, activity was defined as the ability to depolarize a valinomycin-induced membrane potential of liposomes (14). To date, the purified 5-kDa amoebapore has not been demonstrated to have cytotoxic activity for nucleated cells.

Functional screening of an *E. histolytica* genomic library has recently been used to identify several open reading frames that, when expressed in *Escherichia coli*, have hemolytic activity (11). These open reading frames are encoded on a 25-kb amebic ribosomal DNA episome and have no obvious sequence similarities to known proteins. However, it is premature to conclude that any of these open reading frames function in *E. histolytica* to encode hemolysins until there is direct evidence that these sequences are transcribed and translated in *E. histolytica*. Thus far, evidence of their translation is the weak reactivity of the bacteria containing the expressed clones with antiserum produced against whole amebae.

Calcium-dependent and calcium-independent phospholipase A activities identified in amebic extracts have been implicated in cytolysis. Amebic cytotoxicity, but not adherence, was inhibited by Rosenthal's inhibitor and phosphatidylcholine, antagonists of phospholipase A_2 (40). The calcium-dependent phospholipase activity was localized to the plasma membrane surface, the relevant site of amebic cytolytic activity. One possible role for amebic phospholipase A enzymes in cell killing might be in the disruption of target cell membranes, rendering the cell permeable to attack by other amebic enzymes or toxins (16). Because Rosenthal's inhibitor and phosphatidylcholine may act by multiple mechanisms, however, the evidence supporting a role for phospholipase A enzymes in amebic cytotoxicity is, at best, indirect.

Target cells killed by *E. histolytica* undergo DNA fragmentation patterns characteristic of apoptotic death. DNA fragmentation was blocked with Gal or antilectin antibodies, suggesting that the Gal/GalNAc adherence event may initiate a process of programmed cell death in the target cell (34).

Proteolytic activities of *E. histolytica* are believed to be involved in damage of cells and the extracellular matrix of the host. Secreted amebic cysteine proteases cause a cytopathic (as opposed to cytotoxic) effect manifested by cells being released from monolayers in vitro without cell death (24, 45, 57). Expression of a secreted amebic 56-kDa cysteine proteinase has been correlated with the potential of clinical isolates of *E. histolytica* to produce invasive disease, because the cysteine proteinase was identified by gelatin substrate gel electrophoresis in 10 of 10 isolates from patients with colitis or amebic liver abscesses but in only 1 of 10 isolates from asymptomatic patients (44). The purified protein was able to degrade type I collagen, fibronectin, and laminin and caused a loss of adhesion of mammalian cells in culture. The proteinase inhibitor *Z*-phenylalanyl-alanyl-CH_2F inhibited the ability of the amebae to cause the loss of cellular adhesion (12). These results

supported the hypothesis that expression and release of the cysteine proteinase may be involved in the amebic cytopathic effect by its degradation of cell anchoring proteins.

THE LECTIN APPEARS TO BE INVOLVED IN CELL SIGNALING

Antilectin monoclonal antibodies (MAb) and Gal block cytotoxicity even after adherence has occurred (36, 48). These results indicate a role for the lectin in cell killing that is distinct from its adherence function. The purified lectin was not cytotoxic as assessed by ^{51}Cr release (48), suggesting a signal transduction role for this molecule in amebic killing. The deduced amino acid sequences of the putative cytoplasmic domains of each of the three sequenced genes encoding the 170-kDa subunit revealed several potential phosphorylation sites, suggesting that signal transduction may occur by way of phosphorylation (21, 33, 55).

Further implicating the lectin in the signaling process was the finding that polymerized actin appeared at the contact interface of amebic trophozoites and Gal-glycolipid-containing liposomes (2). Glycoprotein-conjugated latex beads, however, failed to stimulate actin polymerization, suggesting that both adherence via the Gal/GalNAc-specific lectin and interaction with lipids of the target membrane bilayer are necessary for amebic activation (as measured by actin polymerization) (1).

LECTIN-MEDIATED RESISTANCE TO COMPLEMENT LYSIS

The human complement system is an important early host defense against infection. *E. histolytica* activates the complement system but is resistant to killing by complement C5b-9 membrane attack complexes deposited on the membrane surface. Components of the amebic plasma membrane that mediate resistance to human complement C5b-9 were identified by screening for neutralizing MAb. An antiamebic MAb was identified that increased *E. histolytica* lysis by the membrane attack complex (at the steps of C8 and C9 assembly). Unexpectedly, the MAb was directed against an epitope contained in the cysteine-rich domain of the Gal/GalNAc lectin. Inhibition of the lectin with 25 mM Gal caused a modest increase in C5b-9 lysis of trophozoites, suggesting that carbohydrate binding activity may contribute to the lectin's ability to block assembly of the membrane attack complex. The purified lectin bound C8 and C9 and conferred C5b-9 resistance to sensitive amebae (attenuated and rendered C5b-9 sensitive by prolonged in vitro cell culture) upon reconstitution. These activities of the lectin were inhibited by the antilectin MAb (3). In this regard, the Gal/GalNAc lectin seems to share functional similarity with the human complement regulatory protein CD59, which also binds the C8 and C9 subunits of the C5b-9 complex (45). In addition to the functional similarities, the lectin shares sequence similarities and antigenic cross-reactivity with CD59 (3). Current work in our laboratories is aimed at understanding the regulation of induction of, and the role of the different lectin heterodimers in, the complement resistance phenotype.

STRUCTURE OF THE Gal/GalNAc LECTIN

The 260-kDa amebic protein purified by Gal affinity chromatography or by adherence-inhibitory MAb affinity chromatography was independently shown to be the Gal/GalNAc lectin in several ways. The purified protein bound to CHO cells in a Gal-inhibitable manner and competitively inhibited amebic adherence to preexposed CHO cells in a dose-dependent manner (31, 39, 47). The antiamebic MAb which blocked adherence recognized the Gal affinity-purified lectin (31). Finally, mouse antiserum produced against the purified protein, but not preimmune sera, blocked adherence to CHO cells by 100% (27). The 260-kDa lectin dissociates into heavy (170-kDa) and light (35-31-kDa) subunits upon reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23).

PRIMARY STRUCTURE OF THE LECTIN HEAVY SUBUNIT

Three genes (*hgl1* to *hgl3*) encoding the Gal/GalNAc-specific lectin 170-kDa subunit have been sequenced from a λZAP genomic library of *E. histolytica* (21, 33, 55). The deduced amino acid sequences of *hgl1* and *hgl3* are 89.2 and 89.4% identical to *hgl2*, respectively, and 95.2% identical to each other. The number and location of every cysteine residue are conserved in each gene (20). All 16 potential N-linked glycosylation sites present in *hgl1* are conserved in *hgl3*, while *hgl2* retained only nine of these sites (33). Analysis of the deduced amino acid sequences of *hgl1* to *hgl3* indicates that the heavy-subunit gene products are transmembrane proteins containing at least four structural domains. *hgl1* to *hgl3* encode amino-terminal signal sequences, supporting the location of the 170-kDa protein isoforms on the cell surface. The amino termini of the mature proteins (amino acids 1 to 187) are relatively cysteine- (3.2%) and tryptophan- (2.1%) rich and hydrophilic. Residues 188 to 378 lack cysteine residues and contain alternating hydrophobic and hydrophilic regions. Residues 379 to 1209 are extremely cysteine-rich (10.8%) and are hydrophilic. Residues 1210 to 1235 are hydrophobic and most likely represent transmembrane domains. The 41 carboxyl-terminal amino acids are relatively hydrophilic and form the putative cytoplasmic domains (21, 33, 55). Fluorescence-activated cell sorter analysis with MAb specific for the cysteine-rich domain of the 170-kDa subunit bound to intact trophozoites, verifying the surface location and membrane orientation of the lectin 170-kDa subunit (19).

Northern (RNA) blot analyses of *E. histolytica* RNA using gene-specific oligonucleotides from the three sequenced heavy-subunit genes all identified mRNAs of 4.0 kb, indicating that all three genes are expressed in *E. histolytica* and that the various messages are identical in size (21, 33, 55). The size of the mRNA is large enough to account for the 3,847 bases in the open reading frame of the cDNA encoding *hgl1* and is consistent with the observations of others that the 5′ and 3′ untranslated regions of mRNAs in *E. histolytica* are very short. Northern blot analysis indicated that the abundance of mRNAs encoding *hgl1* to *hgl3* decreased substantially as the amebae passed from log to stationary phases when grown in liquid culture medium (33). This finding is interesting in light of earlier studies demonstrating decreased ability of late-log and stationary-phase amebae to adhere to, lyse, and phagocytose target cells (25).

PRIMARY STRUCTURE OF THE LECTIN 35-kDa SUBUNIT

The two light subunit genes (*lgl1* and *lgl2*) sequenced are 80.6% identical and have calculated molecular masses of 32-kDa polypeptides with hydrophobic amino- and carboxy-terminal signal sequences (22, 23, 56). Each gene contains two putative N-linked glycosylation sites, although only one site is common to both. FASTA searches of the deduced amino acid

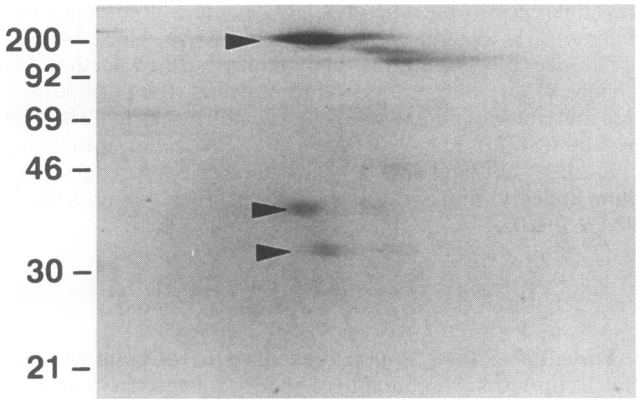

FIG. 2. Two-dimensional SDS-PAGE of affinity-purified lectin. The purified lectin was electrophoresed in a 6% tube gel under nonreducing conditions in the horizontal dimension and in a 10% slab gel under reducing conditions in the vertical dimension. Staining was performed with Coomassie blue. Arrowheads indicate the 170-kDa heavy subunit and the 31- and 35-kDa isoforms of the light subunit. Molecular mass markers in kilodaltons are indicated on the left for the second dimension. Reprinted from reference 23 with permission of the publisher.

sequences revealed no significant similarities to other proteins in the National Biomedical Research Foundation data bank (26). Fifteen residues from the carboxyl terminus is a putative glycosylphosphatidylinositol (GPI) anchor cleavage/addition site (8). The amino acid sequences encoded by *lgl1* and *lgl2* alone are apparently not sufficient to signal GPI addition, however, because unlike the 31-kDa isoform, the 35-kDa isoform was not metabolically labeled with myristic or palmitic acids (23).

The amino acid compositions and CNBr peptides of the 35- and 31-kDa subunits were similar (23), and antibodies raised against a glutathione-*S*-transferase–35-kDa-subunit fusion protein cross-reacted with both isoforms on Western blots (immunoblots) (22), indicating similar structures for the two polypeptides. Differences between the two light-subunit isoforms appear to include carbohydrate and lipid modifications, which may account for their different apparent molecular masses from the 32-kDa mass predicted from the cDNA sequence. Nitrous acid deamination of the [^{3}H]palmitate-labeled 31-kDa isoform released acyl-phosphatidylinositol, supporting the presence of a GPI anchor on the 31- but not 35-kDa isoform (23). The biochemical analyses to date indicate that the primary structure of the lectin is unique, with GPI-anchored and transmembrane subunits.

STRUCTURES OF THE LECTIN HETERODIMERS

Two-dimensional SDS-PAGE of the purified lectin, with the first dimension nonreducing and the second dimension reducing, identified at least two major lectin heterodimers, one containing the 170-kDa subunit with the 35-kDa light-subunit isoform and another containing the 170-kDa subunit with the 31-kDa isoform. Minor heterodimers were seen with 160- and 150-kDa heavy-subunit isoforms associating with the 35- and 31-kDa light-subunit isoforms (Fig. 2). Additional structural complexity almost certainly exists, as a minimum of three different heavy-subunit genes and two different light-subunit genes are expressed in trophozoites. The functional consequences of this structural complexity are under investigation.

Analysis of the native Gal/GalNAc-specific lectin by gel filtration on a Sephacryl S-300 column resulted in elution of the protein as a broad peak at approximately 660 kDa with a shoulder at 440 kDa. These results suggested that the adherence lectin exists in aggregate form (27). Oligomerization of the lectin may play an important role in adherence and amebic pathogenesis as it likely contributes to the glycoside clustering effect (13) by more tightly and rigidly organizing the lectin subunits on the membrane surface.

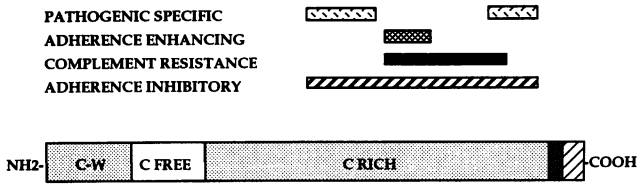

FIG. 3. Locations of the epitopes recognized by 170-kDa subunit-specific MAb as determined by reactivity with deletion constructs of the 170-kDa subunit. The locations of MAb epitopes with different functional effects on the lectin are depicted by boxes above the domain structure diagram of the 170-kDa subunit. The domains of the 170-kDa subunit are the amino-terminal cysteine- and tryptophan-rich domain (C-W, amino acids 1 to 187), the cysteine-free domain (C-free, amino acids 188 to 378), the cysteine-rich domain (C-rich, amino acids 379 to 1209), the transmembrane domain (amino acids 1210 to 1235), and the carboxy-terminal cytoplasmic domain (amino acids 1236 to 1276). Adapted from reference 19 with permission of the publisher.

No classical carbohydrate-binding sequence motifs (7, 17) have been identified in the sequences of heavy and light subunits, leaving the location of the carbohydrate recognition domain of the lectin unknown. Adherence-inhibitory and -enhancing MAb epitopes have been mapped to distinct regions of the cysteine-rich domain of the heavy subunit (see below). Polyclonal antibodies and MAb against the light subunit in contrast do not affect adherence (23a). Recently, it has also been reported that the 170-kDa subunit expressed in vitro had Gal-specific binding activity which was lost when the cysteine-rich domain was deleted (58). These data together suggest that the cysteine-rich region plays a role in carbohydrate binding, either containing or being required for the function of the carbohydrate recognition domain. Direct cross-linking of the lectin with Gal-terminal oligosaccharides is under way to directly identify the carbohydrate-binding domain.

MAPPING OF MAb EPITOPES

Antibodies to different epitopes on the lectin 170-kDa subunit block or enhance Gal-binding activity and block cytolysis and complement C5b-9 resistance. In addition, these MAb distinguish the lectin of pathogenic *E. histolytica* from the lectin of nonpathogenic *E. dispar*, forming the basis of a rapid stool antigen detection test for the diagnosis of amebiasis (10). To define and localize these antibody epitopes on the 170-kDa subunit, DNA sequences encoding portions of *hgl1* were expressed in *E. coli* as glutathione-*S*-transferase fusion proteins (19). Additional fusion proteins containing carboxy-terminal deletions in the lectin were also constructed to roughly map the locations of the seven different MAb epitopes. Adherence-enhancing and -inhibiting MAb epitopes were located within the extracellular cysteine-rich region (Fig. 3), suggesting that this may be the location of the carbohydrate recognition domain. Interestingly, epitopes where complement-inhibitory MAb bound were demonstrated to be near or actually overlap adherence-inhibitory epitopes as well as the

portion of the cysteine-rich domain with sequence identity to the human complement inhibitor CD59 (Fig. 3). Colocalization of the adherence and serum resistance-inhibitory epitopes suggests that these two activities of the lectin may be mediated by the same or closely located regions of the heavy subunit.

THE Gal/GalNAc LECTIN IS A POTENTIAL VACCINE CANDIDATE

The central roles of the Gal/GalNAc lectin in adherence, cytolysis, and complement resistance suggest that it would be a logical vaccine candidate. Theoretically, an antiamebic vaccine that prevented adherence to the colonic mucosa could prevent not only disease but colonization. Cell-mediated and humoral immune responses specific for the Gal/GalNAc lectin have been detected in most immune individuals (29, 49, 52). The Gal/GalNAc lectin has also been shown to be a protective antigen in a gerbil model of amebic liver abscess. Complete protection against amebic liver abscess formation was observed in 67% of animals immunized with MAb-affinity-purified lectin (30), and passive transfer of antilectin antibodies elicited partial protection (43). Several laboratories are investigating the lectin as a subunit vaccine with promising results (28).

CONCLUSIONS AND FUTURE DIRECTIONS

The Gal/GalNAc-specific lectin is a multifunctional protein whose activity is crucial for the cytotoxic activity of *E. histolytica*. It is an unusual protein both functionally and biochemically. The Gal/GalNAc lectin has been shown not only to mediate amebic adherence to a variety of target cells but also to function in cytolytic activity and inhibit the activity of the human complement C5b-9 membrane attack complex. The availability of specific MAb has helped elucidate the role of the lectin in amebic pathogenesis, although the function of the light subunit remains an enigma. The availability of DNA clones of each subunit and the recent development of a DNA transfection system (32) now open the avenue towards a direct genetic analysis of the lectin's role in pathogenesis. Interest in the Gal/GalNAc lectin has increased considerably in recent years, with continued study offering the promise of new strategies for the prevention of an important disease of the developing world.

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