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Etymology of Loa

It has recently been called to our attention that the word 'loa', used for centuries, first by Africans and later by parasitologists, to refer to the 'eye worm'¹, also appears in the terminology of voodoo or Vodun where it refers to a large pantheon of deities that may possess one's soul or being². The word 'loa' may be derived from the Yoruba word 'lawo' meaning 'mystery'³, but according to Bourguignon its origin remains uncertain⁴. To the devotees of vodun, the noon hour, when the sun casts no shadow, is a perilous time⁵. A man without a shadow is a man without a soul and therefore vulnerable to

possession by such spirits as 'loa'. To ward off these spirits, believers wear amulets and cast spells⁶. African vodun evolved in Benin, formerly Dahomey, and was brought to Haiti with slavery during the seventeenth and eighteenth centuries⁷.

Since both adults and microfilariae of the 'eye worm' *Loa* are diurnal, with maximum activity occurring at noon (when the West African would be most susceptible to spirit possession), and since West African vodun and the 'eye worm' share a common geographic origin, we have reached the tentative conclusion that one is probably the etymological source for the other. Whether the helminthological *Loa* predated the anthropological one remains a matter of conjecture.

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Focus

Cell Surface Proteins of *Entamoeba histolytica*

B.J. Mann and W.A. Petri, Jr

To ask what is new in *Entamoeba histolytica* research, one need look no further than the surface of this protozoan parasite. In the past year the cloning and partial characterization of five different surface antigens have been reported, a remarkable result of international research efforts against amebiasis. One of these proteins is the first protective immunogen identified in the animal model of amebic liver abscess. Barbara Mann and William Petri review these recent results, propose a nomenclature for the gene family of *E. histolytica* galactose lectins and discuss the roles of the different surface proteins in adhesion.

Near the turn of the century a 29-year-old physician sought medical care for prolonged dysentery and fever. Before returning to the United States, he had resided in Panama for almost six years. Sir William Osler examined the patient and noted that:

His general condition was very good, considering that he had had severe dysentery and an irregular fever for more than two months. The liver was slightly enlarged anteriorly but not specially sensitive. Posteriorly, there seemed to be a very distinct extension of the dulness [sic] upward. He had six or eight mucoid stools with traces of blood daily. I saw him subsequently on four occasions and the symptoms remained practically the same. The temperature rose each day to about 103°...

The suspicion entertained at first that he had abscess of the liver was gradually confirmed, and on March 22nd Dr Tiffany aspirated, and then incised and drained two large abscess cavities in the right lobe of the liver. The pus was thick, of creamy consistency, in color, in places slightly bile-stained, but it has not the reddish-brown and anchovy-sauce-like appearance presented by the pus in many cases of hepatic abscesses.

I made an examination of the pus at the Biological Laboratory, within three-quarters of an hour of its withdrawal and found in it, in large numbers, the amoebae which Kartulis had described.

After the operation the dysenteric symptoms did not abate in the slightest; he continued to have from eight to sixteen movements daily. On each day there were found in these stools many characteristic examples of the amoebae... The general character of the amoebae corresponded in every particular with those found in the liver.

Osler concluded that:

It is impossible to speak as yet with any certainty as to the relation of these organisms to the disease. The subject is deserving of extended study.

The patient died on 5 April¹. It was one year later, in 1891, that Osler's colleagues Councilman and Lafleur published the key paper 'Amoebic Dysentery' in the

*Johns Hopkins Hospital Report*². In it they described the pathology of amebic colitis and liver abscess, including the invasion through the intestinal epithelium by the amoebae, and emphasized the lack of contribution of intestinal bacteria to the colitis and liver abscess.

The occasion of the 100th anniversary of this landmark monograph is an opportune time to review recent advances in the study of this protozoan that remains a worldwide cause of human suffering. We will arbitrarily limit our discussion to the five surface proteins whose sequences have been reported, and not cover other potentially important but less well-characterized surface molecules that have been described, such as the fibronectin receptor³, neuraminidase⁴, phospholipase A⁵, surface sialoglycoproteins⁶, the 112 kilodalton (kDa) adhesin⁷, the chitotriose lectin⁸ and the 30 kDa^{9,10} and 90 kDa¹¹ surface antigens.

Serine-rich *E. histolytica* Protein

A serine-rich protein has been identified by Stanley and colleagues by differential hybridization screening of an *E. histolytica* complementary (c)DNA library¹². The library was constructed from *E. histolytica* strain HMI:IMSS and screened with radiolabeled cDNA from HMI:IMSS and the non-*E. histolytica* Laredo strain. Several clones that hy-

bridized with HMI:IMSS and not Laredo cDNA were identified and one 0.7 kilobase (kb) cDNA sequenced. This *E. histolytica*-specific cDNA contains two open reading frames (ORF). Antibodies produced to *trpE* fusion proteins using the first but not the second ORF recognized *E. histolytica* proteins on western blots. Human immune sera and rabbit anti-*E. histolytica* antiserum recognized the fusion protein using ORF1 and not ORF2, which suggests that ORF2 is not translated in the trophozoites. Surprisingly, antibodies raised to the ORF1 fusion protein identify *E. histolytica* proteins, which at 46 and 52 kDa are twice the size predicted from the cDNA sequence (25 kDa). Post-translational modifications have been speculated to be involved but have not been shown to account for the difference in molecular weight. The proteins recognized by the ORF1 fusion protein antiserum were present in a 100 000 g pellet of sonicated trophozoites and therefore are likely to be associated with membranes or vesicles.

The structure of the serine-rich protein encoded by ORF1 shares similarities with the malaria circumsporozoite proteins. The amino terminus contains a 13 amino acid putative signal sequence that is followed by a region of charged amino acids. The center of the protein contains tandem octapeptide and dodecapeptide repeats, with a 13 amino acid hydrophobic carboxy-terminus domain. There are no potential sites for *N*-linked glycosylation.

A function for the serine-rich protein in amebic adhesion has been proposed based on the finding that a 1:10 dilution of antiserum to the ORF1-*trpE* fusion protein inhibits adherence to Chinese hamster ovary (CHO) 1021 cells by 70%. The same investigators have shown in an earlier paper that amebic adherence to this cell line can be inhibited by 78% with *N*-acetyl lactosamine. As the serine-rich protein is distinct from the *E. histolytica* galactose and *N*-acetyl lactosamine binding adhesin (see below), the effect of the ORF1 fusion protein antiserum on adherence may have been indirect, leaving the function of the serine-rich protein yet to be defined.

Cysteine-rich 29 kDa Surface Antigen

Screening of an expression cDNA library (constructed from *E. histolytica* strain H-302:NIH) with rabbit polyclonal anti-*E. histolytica* serum by Torian and colleagues has resulted in the isolation

of a 700 base pair (bp) cDNA insert³. However, this cDNA is incomplete, lacking a portion of the 5' end of the copied messenger (m)RNA. The amino acid composition of the protein encoded by the cDNA contains 7% cysteine, and monoclonal and polyclonal antibodies have been produced to this cysteine-rich protein by expressing it as a fusion protein with the carboxy terminus of glutathione *S*-transferase. Monoclonal antibodies were used to show that the 29 kDa protein is expressed on the surface by indirect immunofluorescence.

This cDNA hybridizes on northern blots to an RNA molecule of 1050 bp and to DNA extracts from four different axenic strains of *E. histolytica* although not to *E. invadens*, *Acanthamoeba castellanii* or *Trichomonas vaginalis*. The monoclonal antibodies also recognize the cysteine-rich protein in the axenic strains as well as six clinical isolates of pathogenic and nonpathogenic zymodemes of *E. histolytica*. Not all of the monoclonal antibodies recognize all of the clinical isolates, indicating that there are epitopes that are not conserved in all strains.

The unusual cysteine content of the protein may offer some clues to its function. At the 5' end of the cDNA there is the motif C₂-X₃-C-X₄-C₂-X₂-C₃, where X is any amino acid. A similar sequence was found in inverse order in the peplomer protein E2 of porcine corona virus, which is involved in viral binding and entry into the cell, and in a wool keratin protein that is thought to impart structural rigidity. The monoclonal antibodies produced against the 29 kDa cysteine-rich antigen by Torian et al.¹³ should prove to be powerful tools to reach a further understanding of the antigen's structure and function.

Variable 125 kDa Surface Antigen

A 125 kDa protein band is recognized by the immune sera of 73% of patients with amebic liver abscess. Edman et al. used pooled human immune sera to isolate a cDNA clone encoding this antigen from the HMI:IMSS strain¹⁴. Sequence analysis of the cDNA reveals it to be identical to the partial sequence of the cEH-P1 protein reported by Tannich et al.¹⁵ Monospecific antibodies, as well as a monoclonal antibody prepared from mice immunized with the 125 kDa band cut out of gels, have been used to demonstrate the cell surface location of the 125 kDa antigen by indirect immunofluorescence.

The complete amino acid sequence was derived from a genomic clone that hybridizes with a mRNA of 3.0 kb, which lacks introns and encodes a protein of 125 513 Da. This sequence is rich in tyrosine and asparagine residues, contains 17 potential *N*-linked glycosylation sites and has a 35 amino acid amino-terminus hydrophobic region which is postulated to function as a signal/membrane anchor; there are no other hydrophobic domains in the protein. A 31 amino acid area has been found to have limited sequence identity with the β_1 chain of mouse integrin, the β chain of the human fibronectin receptor and the band 3 precursor of chicken integrin, but the functional significance of the sequence identity is unclear.

The sequences of a 470 amino acid portion of 125 kDa antigen in pathogenic and nonpathogenic *E. histolytica* have been compared¹⁴. The 125 kDa antigen sequence of the pathogenic HMI:IMSS strains reported by Edman et al.¹⁴ and Tannich et al.¹⁵ in this region differ by five amino acid substitutions (1.0%). At least three of the five substitutions involve more than one nucleotide change and are therefore unlikely to represent reverse transcriptase or sequencing artefacts. Comparison of two nonpathogenic strains (SAW 1734 and REF 291) reveals six amino acid substitutions (1.3%). However, 61 amino acid substitutions (12.9%) have been seen when the sequence from the pathogen is compared to that of the nonpathogen¹⁴.

The two 125 kDa antigen sequences from nonpathogenic strains are thus more similar to one another than to the sequences from the pathogenic strain HMI:IMSS. Tannich et al. had earlier shown unique Southern blot patterns for the 125 kDa antigen in pathogenic and nonpathogenic strains for nine different *E. histolytica* strains¹⁵. They recently extended these studies to an additional 48 strains, and only one of the pathogenic strain had a nonpathogenic restriction pattern^{15,16}. These analyses of the 125 kDa antigen gene support the concept that pathogenic and nonpathogenic *E. histolytica* strains are genetically distinct.

Galactose Lectin

The galactose lectin is an interesting molecule to study by virtue of its central role in adhesion and contact-dependent cytolysis. There is considerable evidence to support a critical role for a galactose lectin in pathogenesis. Ravdin and Guerrant have shown that

adherence to, and killing of, CHO cells by *E. histolytica* are completely blocked by galactose or *N*-acetyl-D-galactosamine¹⁷. Phagocytosis of bacteria and erythrocytes, and binding to human colonic mucins can also be blocked by galactose^{17,18}. Finally, CHO cell mutants lacking terminal *N*-acetyl lactosamine residues have been found to be resistant to amebic adherence and killing^{19,20}. Purified galactose lectin binds to CHO cells and competitively inhibits amebic attachment to CHO cells in a galactose-sensitive manner²¹. Moreover, antiserum raised against the purified galactose lectin inhibits amebic adherence to CHO cells by 100% (Ref. 22) and blocks the binding of human colonic mucin glycoproteins to amebic trophozoites¹⁸.

The galactose lectin is a 260 kDa heterodimer consisting of a 170 kDa subunit and a 35 kDa subunit linked by disulfide bonds²². Polyclonal antisera and monoclonal antibodies raised against the native or denatured lectin recognize only the 170 kDa subunit and it appears that the 35 kDa subunit is not immunogenic in humans, mice, rabbits or gerbils²². The light subunit has been shown to bind fibronectin on western blots (B.J. Mann and W.A. Petri, Jr, unpublished), and may be the fibronectin receptor identified by Talamas-Rohana and Meza³. However, owing to the lack of specific antibody against the 35 kDa subunit, this has not yet been shown for intact trophozoites.

Monoclonal antibodies specific for the 170 kDa subunit block or enhance amebic adherence to CHO cells and colonic mucins, suggesting that the adherence domain resides within the heavy subunit²³. The ability of anti-lectin antibodies to dramatically increase the galactose-binding activity of the lectin indicates that its activity could be subject to conformational control. These same antibodies distinguish pathogenic from nonpathogenic strains of *E. histolytica*. All six epitopes defined by the murine monoclonal antibodies were present on the 16 pathogenic strains tested but only two of these six epitopes were present on the nonpathogenic strains²⁴. The presence of the galactose lectin in nonpathogenic strains may reflect its requirement for amebic co-oxidation of the large bowel, because of its function as the receptor for human colonic mucins.

The Galactose Lectin as a Protective Antigen

Primates and humans are the only known reservoirs of *E. histolytica*. An

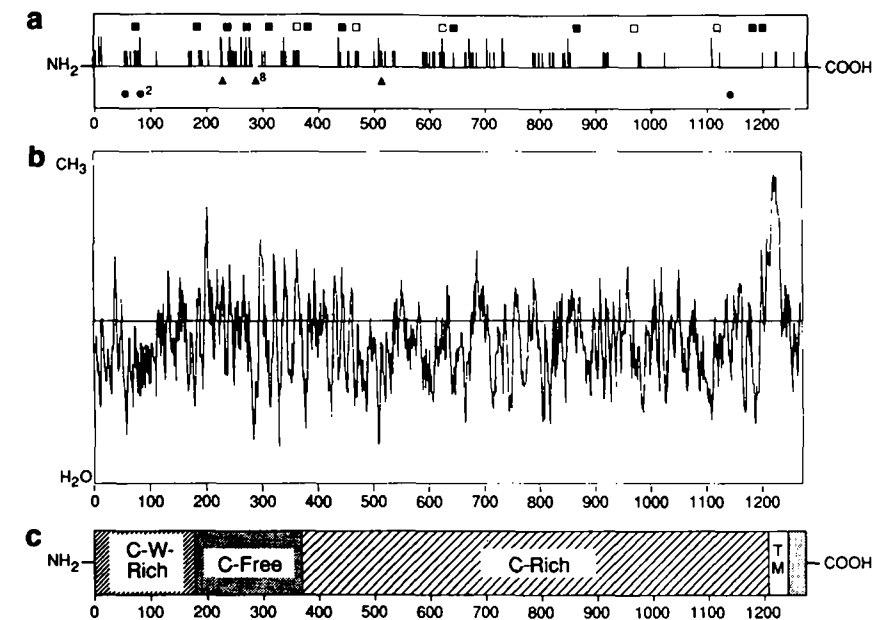


Fig. 1. A comparison of two different genes encoding the galactose lectin 170 kDa subunit. (a) Amino acid differences in hgl2 compared to hgl1. Residue numbers refer to hgl1. Variant amino acids are depicted by vertical lines at the residue number in hgl1. Conservative amino acid changes are indicated by short vertical lines. Nonconservative changes are depicted by tall vertical lines. Insertions in hgl2 with respect to hgl1 (closed circles) and deletions in hgl2 with respect to hgl1 (closed triangles) are indicated. The number to the right of the symbols indicates the number of residues inserted or deleted. Glycosylation sites in both hgl1 and hgl2 (closed squares) and only in hgl1 (open squares) are shown. (b) A hydrophobicity plot of the derived amino acid sequence of hgl1. Residue numbers refer to hgl1. (c) The putative structural domains of hgl1 and hgl2. Cysteine- and tryptophan-rich (C-W-rich); cysteine-free (C-free); cysteine-rich (C-rich); transmembrane domain (TM).

anti-amebic vaccine that prevents large bowel colonization could theoretically eliminate *E. histolytica* as a cause of human disease. The galactose lectin has several properties that make it an attractive subunit vaccine candidate: (1) as mentioned, the galactose lectin appears to play a critical role in amebic adherence to colonic mucosa, in the phagocytosis of bacteria and erythrocytes and in the initiation of contact-dependent cytotoxicity; (2) anti-lectin antibodies that prevent these functions could potentially protect the host from invasion and (3) the galactose lectin is a major amebic antigen recognized by 95% of all human immune sera tested²⁵, and is antigenically conserved among geographically distinct isolates of *E. histolytica*^{24,26}.

The ability of the galactose lectin to elicit a protective immune response has been tested in the gerbil model of amebic liver abscess. Gerbils received three immunizations with the affinity-purified lectin injected subcutaneously or intraperitoneally in Freund's adjuvant before direct intrahepatic challenge with 5×10^5 *E. histolytica* trophozoites. All of the immunized gerbils developed prechallenge anti-lectin antibody titers of 1:1024 or greater, and no anti-lectin antibodies were detected in the undiluted sera from sham-immunized

animals. The prechallenge immune sera completely blocked adherence at a 1:10 dilution but increased adherence to 163% of control levels at a 1:1000 dilution. Complete protection from liver abscess formation was seen in the majority of the lectin-immunized animals in three trials with subcutaneous immunization and one with intraperitoneal immunization. Combining the data from the four trials, 81% of control and 27% of immunized gerbils developed liver abscesses for an overall vaccine efficacy of 67% (Ref. 27).

Galactose Lectin Gene Family

The molecular cloning and sequencing of the 170 kDa subunit of the galactose lectin from pathogenic *E. histolytica* has recently been reported by our laboratory (B.J. Mann et al., abstract*) (Ref. 28) and Tannich et al.²⁹ A comparison of the amino acid sequences described by these two groups revealed that they are only 87.6% homologous (Fig. 1a), suggesting the presence of at least two genes encoding the

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170 kDa subunit. The presence of more than one gene encoding the 170 kDa subunit has been suggested by the observed microheterogeneity in the amino acid sequences obtained by sequential Edman degradation of the amino terminus and cyanogen bromide (CNBr) fragments²⁸, and by the complex restriction fragment patterns seen on Southern blots probed with the cDNA for the 170 kDa subunit²⁹. The copy number of the genes encoding the 170 kDa subunit and their genomic organization have yet to be determined. The sequences reported in Fig. 1, which represent two different genes encoding the 170 kDa subunit of the galactose lectin, are proposed to be called *hgl1* (Ref. 28) and *hgl2* (Ref. 29) for heavy subunit galactose lectin. *hgl2* is a cDNA clone isolated from the pathogenic strain HMI:IMSS. The DNA encoding the first 482 amino acids of *hgl1* was isolated from the HMI:IMSS genomic DNA with the remainder of the gene sequence determined from a cDNA clone of pathogenic strain H-302:NIH. A combination of sequencing and polymerase chain reaction (PCR) experiments has revealed that *hgl1* does not contain any introns²⁸.

The calculated relative molecular masses of *hgl1* and *hgl2* are 143 241 Da and 143 780 Da, respectively, and their individual amino acid composition and overall structure are also very similar. The nonuniform distribution of hydrophobic amino acids of *hgl1* and *hgl2* suggests the existence of five structural domains (Fig. 1b, 1c): (1) At the amino terminus of the mature protein is a 187 amino acid cysteine (3.2 mol %) and tryptophan (2.1 mole %)-rich amino-terminus domain; (2) The second cysteine-free domain, corresponding to *hgl1* residues 188–378, contains alternating hydrophobic and hydrophilic stretches of amino acids and is the most variable between the two proteins. A substantial nonconservative change is the addition of eight amino acids at amino acid 285 in *hgl1*; (3) The third structural domain is the cysteine-rich domain, which contains 10.8 mol % of cysteine. The number and position of the cysteine residues have been conserved with the exception of amino acid 804, which is a serine residue in *hgl2*. It has been shown that the cysteine residues play an important role in the observed protease resistance of the 170 kDa subunit and may be important for parasite survival in the gut²⁸; (4) A putative transmembrane domain contains only two conservative changes between *hgl1* and *hgl2* and (5) The carboxy-terminal domain is a putative

cytoplasmic tail that is also highly conserved. There are a total of 11 threonine, serine and tyrosine residues that are potential sites for phosphorylation in the cytoplasmic domain of *hgl2* and only nine such residues in *hgl1*. Tyrosine residue 1261, present in both *hgl1* and *hgl2*, is surrounded by an amino acid sequence that shares identity with the autophosphorylation site of the epidermal growth factor receptor²⁸. It is interesting to speculate that phosphorylation of the carboxy-terminal domain may be a mechanism of activation of the lectin, and that the degree of phosphorylation could functionally distinguish the different forms of the 170 kDa subunit. The high degree of conservation of the cytoplasmic domain also suggests that the lectin may interact with a cytoplasmic protein in another route of signal transduction.

The 170 kDa subunit has been shown to be glycosylated and the treatment of amebae with tunicamycin results in almost a complete loss of adherence to CHO cells, showing that there is a critical role for *N*-linked glycosylation in amebic adherence. Sixteen potential glycosylation sites have been found in *hgl1* (Fig. 1c) and *hgl2* has only nine sites, although all of these sites are conserved in *hgl1*. The difference in the number of potential glycosylation sites could also indicate that the different members of the lectin gene family are functionally distinct.

The carbohydrate-binding portion of the molecule has not yet been identified and the 170 kDa sequence does not share any significant amino acid identities with the conserved regions of C- or S-type lectins, the *E. coli* α -1,4-galactose-binding pilus or plant lectins that have been sequenced thus far²⁸. Although the evidence suggests that the carbohydrate-binding activity resides in the 170 kDa subunit, it is possible that it is contained within the 35 kDa subunit or is formed by the heterodimer of the two subunits.

Future Investigations

The recent discovery of two different genes encoding the 170 kDa subunit of the galactose lectin opens up new avenues of investigations. The number of family members, physical association of the gene copies, functional differences and possible transcriptional differences can be examined. A comparison of the similarities and differences between different members of the gene family should provide valu-

able information regarding the adherence, cytolytic and cell-signaling functions of the lectin. The contributions of the light subunit to the functional activities of the galactose lectin remain unresolved as do the biological functions of many of the other surface antigens identified to date. Continued characterization of the galactose lectin and other surface proteins should provide greater understanding of the immunology, pathogenicity and molecular biology of this globally important parasite.

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