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Expression, purification, and evaluation of recombinant LecA as a candidate for an amebic colitis vaccine

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Abstract

Entamoeba histolytica, which causes amebic colitis and liver abscess, is considered a major enteric pathogen in residents and travelers to developing countries where the disease is endemic. Interaction of this protozoan parasite with the intestine is mediated through the binding of the trophozoite stage to intestinal mucin and epithelium via a galactose and N-acetyl-D-galactosamine (Gal/GalNAc) lectin comprised of a disulfide linked heavy (ca. 180 kDa) and light chain (ca. 35 kDa) and a noncovalently bound intermediate subunit (ca. 150 kDa). Our efforts to develop a vaccine against this pathogen have focused on an internal 578 amino acid fragment, designated LecA, located within the cysteine-rich region of the heavy chain subunit because: (i) it is a major target of adherence-blocking antibodies of seropositive individuals and (ii) vaccination with his-tagged LecA provides protection in animal models. We developed a purification process for preparing highly purified non-tagged LecA using a codon-optimized gene expressed in *Escherichia coli*. The process consisted of: (i) cell lysis, collection and washing of inclusion bodies; (ii) solubilization and refolding of denatured LecA; and (iii) a polishing gel filtration step. The purified fragment existed primarily as a random coil with β -sheet structure, contained low endotoxin and nucleic acid, was highly immunoreactive, and elicited antibodies that recognized native lectin and that inhibited in vitro adherence of trophozoites to CHO cells. Immunization of CBA mice with LecA resulted in significant protection against cecal colitis. Our procedure yields sufficient amounts of highly purified LecA for future studies on stability, immunogenicity, and protection with protein-adjuvant formulations.

Keywords

Entamoeba histolytica; Lectin; Amebiasis; Diarrhea; LecA vaccine

1. Introduction

Amebic colitis and liver abscess are due to infection with the enteric protozoan parasite *Entamoeba histolytica*. This parasite has recently been separated using modern diagnostic techniques from the nonpathogenic parasite *E. dispar*, which is more common and identical

in appearance to *E. histolytica* [11]. The World Health Organization (WHO) estimates that approximately 50 million people worldwide suffer from invasive amebic infection each year, resulting in 40–100 thousand deaths annually [28,46]. Carefully conducted serologic studies in Mexico, where amebiasis is endemic, demonstrated antibodies to *E. histolytica* in 8.4% of the population [7]. In the urban slum of Fortaleza, Brazil, 25% of the people tested carried antibody to *E. histolytica* and prevalence of anti-amebic antibodies in children aged 6–14 was 40% [5]. A prospective study of preschool children in a slum of Dhaka, Bangladesh demonstrated new *E. histolytica* infection in 39% of children >1 year, with 10% having an *E. histolytica* infection associated with diarrhea and 3% with dysentery [17]. Amebiasis also is the second most common cause of diarrhea in returning travelers [14]. Recently the Global Enteric Multicenter Study (GEMS) has demonstrated *E. histolytica* infection to be among the top 15 pathogens resulting in severe diarrhea in infants and children in Africa and Asia [23]. Thus, there is an increasing recognition of the burden of infection due to this protozoan parasite.

E. histolytica invades tissue and causes clinical disease following ingestion of the infectious cyst form of the parasite from fecally contaminated food or water [2,9,34,35,42]. Excystation of the amebic trophozoites occurs in the intestinal lumen. Trophozoites adhere to the colonic mucus and epithelial cells through interaction of a Gal/GalNAc-specific lectin [29,31]. The trophozoite kills host epithelial and immune cells in a process that requires the Gal/GalNAc lectin. *E. histolytica* resists the host's immune response and survives to cause extra-intestinal infection such as amebic liver abscess.

Several different *E. histolytica* proteins have been studied as potential vaccines. These include the serine-rich protein, a 29-kDa cysteine-rich antigen, and the Gal/GalNAc-specific lectin [37–41]. The Gal/GalNAc-specific lectin has been examined in the greatest detail and results support its evaluation as a potential vaccine candidate [4,21,22,36,37]. The LecA domain encompasses the critical neutralizing antibody epitopes for amebic adherence, killing and endocytosis of host cells. In children an IgA antibody response against LecA is associated with immunity. Preliminary studies have demonstrated that the LecA alum-absorbed parenteral vaccine provides protection from amebic colitis in a murine model. Protection provided by the vaccine in mice correlates with the frequency of antigen-specific CD4⁺ T cells that produce intracellular IFN- γ , whereas in children both fecal IgA and IFN- γ , are associated with protection.

The Gal/GalNAc lectin is a 260 kDa heterotrimer of highly conserved disulfide-linked heavy (Hgl) and light (Lgl) subunits non-covalently associated with an intermediate subunit (Igl)[3,10,13,27,30,32,33,43,45]. The carbohydrate recognition domain (CRD) is a cysteine-rich region within Hgl recognized by adherence-inhibitory MAb [25,26]. Native lectin can be purified from *E. histolytica* cultures, but not in amounts sufficient as a vaccine candidate. We have focused on a region located within Hgl designated “LecA” (aa 578–1154) as a vaccine candidate because (i) it is a major target of the cell mediated and humoral immune response in seropositive individuals and (ii) vaccination with a his-tagged version provides protection in animal models [16,19]. We now describe a new scalable purification process for nontagged LecA and demonstrate LecA-mediated protection in a recently developed mouse model of amebic colitis that more accurately mirrors amebic colitis in humans rather than the liver abscess model that was used in past studies.

2. Materials and methods

2.1. Reagents, his tagged LecA, and protein determination

Chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and Fisher Scientific (Waltham, MA) unless otherwise noted. Purification processes were done with ACS or

higher grade chemicals to minimize metal ion contamination. Media preparations were prepared using non-animal based Veggie™ reagents from EMD4 Biosciences (Gibbstown, NJ). His-tagged LecA was prepared as previously described [19]. The Thermo Scientific/Pierce Modified Lowry Assay (Rockford, IL) was used to determine protein concentration [24].

2.2. LecA cloning and expression

The *lecA* clone was codon-optimized and synthesized by DNA2.0 (Menlo Park, CA) in the vector pJexpress401 that contains the kanamycin resistance (Kan^r) gene and a T5 promoter for gene expression. Expression was done in *Escherichia coli* HMS174 (EMD4Biosciences, Gibbstown, NJ). Transformation was performed following the manufacturer's recommendations. 2-L shaking flasks containing 1 L of 2 × YT media + kan (50 µg/mL) were inoculated from an overnight culture, and incubated at 37 °C with shaking. Induction was initiated at OD₆₀₀ of 0.6–0.8 by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and continued for 3 h. Cells were collected by centrifugation, with a typical yield per 1-L of 5.25 ± 0.3 g wet cell weight. Cells were stored frozen at –20 °C.

2.3. Purification

Cell lysis and subsequent washing steps were modified from previously described procedures [8]. Cell pellets were thawed on ice and suspended in Inclusion Body Wash Buffer (IBWB) consisting of 2%CHAPS, 20 mM Tris–HCl, 10 mM EDTA, pH 8.0, at 5 mL per gram wet cell weight. Lysis was performed with sonication and inclusion bodies (IB) were collected by centrifugation. Following lysis, the IB preparation was washed twice with IBWB and twice with an excess of 25% isopropyl alcohol (IPA). IB were solubilized with 7 M guanidine-HCl, 0.15 M reduced glutathione, 0.1 M Tris–HCl, 2 mM EDTA, and 25 mM DTT at pH 8.0, and gently stirred at 4 °C.

Following solubilization, the preparation was clarified by centrifugation. To facilitate refolding, the IB preparation was added dropwise with gentle stirring to a 50-fold volume of 50 mM Tris–HCl, pH 8.0 containing 0.5 M L-arginine and 0.6 mM oxidized glutathione. Refolding was completed overnight at 4 °C without stirring. The refolded sample was brought to room temperature, diluted 1:2 in 1X PBS, pH 7.4, and concentrated 2-fold using Vivaflow 50 crossflow cassettes (30,000 cutoff, Sartorius Stedim North America, Bohemina, NY). The concentration and dilution process was repeated an additional three times. Following the final buffer exchange, the sample was concentrated 6-fold, filtered through a 0.45 µm membrane filter, and concentrated 10-fold using Amicon Ultra-15 Centrifugal filter units (10,000 cutoff, Millipore, Burlington, MA).

The concentrate was applied to a Superdex S-200 XK26/60 column (prep grade, GE Healthcare, Piscataway, NJ) equilibrated with PBS and eluted in the downward flow mode at a flow rate of 2.5 mL/min. Fractions were assayed for A₂₈₀ and A₂₅₄, and analyzed for protein by SDS-PAGE. LecA peak fractions were pooled and stored at 2–8 °C.

2.4. Isoelectric focusing and electrophoresis

LecA was applied to the first dimension ZOOM® strip (Life Technologies, Carlsbad, CA) and electrofocusing was performed at 200 V for 1 h, 450 V for 45 min, 750 V for 45 min, and 2000 V for 1.5 h. The ZOOM® strip was then cast into a NuPAGE® 4–12% Bis-Tris ZOOM® gel and sealed with 0.5% agarose. Staining was done with Bio-Safe™ Coomassie stain. Commercially available NuPAGE® 4–12% Bis-Tris gels were used for SDS-PAGE. LecA was run using NuPAGE® MOPS SDS buffer kit according to the manufacturer's instructions. For Western blots, 7F4 MAbs, 3F4 MAbs, polyclonal antibody against CRD, and

anti-*E. coli* antibodies (Abcam®, Cambridge, MA) were used. Native-PAGE was performed using the Native PAGE™ Novex® Bis-Tris 4–12% acrylamide gel system. Silver staining was performed using the SilverQuest™ kit.

2.5. Characterization studies

The apparent molecular mass was determined by size exclusion chromatography on a Superdex 200 XK26/60 column [1]. Circular dichroism (CD) spectra were measured using an Aviv model 400 spectropolarimeter (Lakewood, NJ) in 0.1 cm cuvettes. Endotoxin levels were determined using the FDA-licensed Endosafe PTS™ cartridges and reader from Charles River Laboratories (Charleston, SC). Results were confirmed by PTS kinetic LAL analysis and kinetic chromogenic testing at Charles River Laboratories. Double-stranded DNA and RNA were quantified using the Qubit™ Fluorometer and their respective kits (Life Technologies). Sequencing analysis was performed at the Protein Sciences Facility at the University of Illinois (Urbana–Champaign). For N-terminal sequence analysis, proteins were transferred to PVDF membranes, and sequencing was performed on a Procise 494 HT Perkin-Elmer instrument using Edman chemistry. Results were analyzed using the NCBI-eukaryotes database. Immunoreactivity with 7F4 MAb, 3F4 MAb, 1G7, 3D12, and anti-CRD antibody (Fig. 1) was determined by endpoint titration with the *E. HISTOLYTICA* II ELISA (TechLab, Inc., Blacksburg, VA). Analysis of best fit curves was determined by simple linear regression. All MAbs have been described previously [25,26].

2.6. Reaction of anti-LecA antiserum with native lectin and LecA

New Zealand white rabbits (female, 5 lb) were injected subcutaneously with 200 µg LecA emulsified in Complete Freund Adjuvant followed two weeks later by boosts at 1-week intervals with 100 µg LecA emulsified in Incomplete Freund Adjuvant. All animal work was approved by the Animal Care Committee at Cocalico Biologicals, Inc. (Reamstown, PA). Reactivity of anti-LecA antiserum was determined by endpoint titration using microwells coated with immobilized native lectin or LecA.

2.7. CHO cell adherence assay

Chinese hamster ovary (CHO) cells were grown and maintained in α -MEM medium, and *E. histolytica* strain HM1:IMSS trophozoites were grown as previously described [12]. The adherence assay was performed as described by Ravdin and Guerrant [35]. *E. histolytica* trophozoites (1×10^4) were preincubated with rabbit preimmune or test serum on ice for 1 h. Trophozoites and CHO cells were then mixed, centrifuged together and incubated on ice for 90 min. Just prior to microscopic analysis, the tubes were vortexed briefly and cells counted on a haemocytometer. Adherence was measured as the number of trophozoites having at least three adherent CHO cells and reported as percent rosettes formation. At least 100 amebae were counted in each tube and each sample was done in triplicate.

2.8. Immunizations, antigen detection, and *E. histolytica* challenge

Four week old male CBA/J mice were obtained from the Jackson Laboratory and maintained under specific-pathogen-free conditions at the University of Virginia. Fifteen mice per group were immunized subcutaneously at weeks 0, 2 and 4 with adjuvant EM014 alone (control group), EM014 + LecA or EM014 + his-LecA (20 µg antigen per mouse per immunization) in a 100 µl final volume [20]. Each mouse received a total of three immunizations at 2-week intervals (weeks 0, 2 and 4). All the mice were challenged intracecally three weeks after the final boost with *E. histolytica* strain HM1: IMSS which had been sequentially passed through the mouse cecum. Mice were challenged with 2×10^6 log phase trophozoites in 150 µl, injected intracecally after laparotomy [18]. The animals were sacrificed one week after the challenge and cecal contents obtained. Typically, 200 µl of cecal contents were used for

amebic determination with the TechLab *E. histolytica* II kit, and the remaining cecal contents were cultured in TYI-S-33 medium for five days at 37 °C.

2.9. Statistical analysis

All statistical analysis involving protection studies was done using GraphPad Prism software. Chi square test was used to compare values for protected and unprotected groups, while Mann–Whitney test was used to compare fecal antigen load values. Significance for the adherence assay was calculated using Student's *t*-test.

3. Results

3.1. LecA expression, purification, and characterization

The engineered organism grown in animal-free Veggie™ yeast extract and peptone gave expression levels comparable to media containing beef products, with >80% of the expressed LecA present as Inclusion Bodies (IB) (data not shown). More than 80% of contaminants were removed by simple washing of IB. Guanidine–HCl effectively solubilized washed IB, with refolding being done using a previously described arginine-assisted process [18]. The refolding process involved dilution of the denatured protein into arginine-containing buffer followed by concentration/dilution steps to incorporate NaCl. Analysis by immunoassay confirmed that refolding had occurred properly. Size exclusion of the refolded, solubilized concentrate removed DNA, small nucleic acids, and aggregated proteins (Fig. 2). The main peak contained LecA and two minor peaks of low MW_r polypeptides. Most of the endotoxin eluted prior to the primary LecA peak.

SDS-PAGE and silver staining showed a major band, confirmed as LecA by N-terminal sequencing and tryptic digest analysis, which had an estimated molecular mass of 70 kDa, in line with the predicted value (Fig. 3). The major band constituted >95% of the Coomassie-stained material. Several minor contaminants, representing RNA polymerase, transcription repair coupling factor, and lac repressor, represented <5% of the total protein. The apparent molecular mass, which was determined with 5 mM dithiothreitol in the sample and 0.5 mM dithiothreitol in the column buffer, was roughly twice the size observed by SDS-PAGE. Monoclonal antibodies and polyclonal antibodies specific for LecA and CRD gave identical patterns by Western blot analysis (Fig. 3). In addition to the conformational mAbs 3F4 and 7F4 already discussed, mAbs with a linear epitope (mAb 1G7) and another conformational epitope (mAb 3D12) gave comparable ELISA results for both native Lectin and LecA (data not shown). Immunoblot analysis with anti-*E. coli* antiserum did not reveal *E. coli* contaminants; however, sequence analyses revealed the three minor *E. coli* contaminants listed above. Analytical native gel electrophoresis showed all three lots to be identical. All three lots had low levels of endotoxin (0.1 EU/μg), both by in-house and external testing, and DNA and RNA levels 82 ng/mg and <20 ng/mg, respectively (Table 1). Simple linear regression analysis showed that immunoreactivity with nontagged LecA was higher than with his-tagged LecA, with values closer to those obtained with native lectin. LecA that had been heat-treated (boiling for 2 min) was no longer immunoreactive. Anti-LecA antiserum reacted with LecA at a titer of 50,000, and with native lectin at a titer of 100,000. LecA stored at 2–8 °C for 18 months or lyophilized retained the same immunoreactivity level and exhibited the same CD spectra as freshly prepared LecA.

3.2. Anti-LecA antiserum prevents parasite adherence to monolayer cells

We determined whether anti-LecA antibodies recognized the parasite in vitro and blocked the parasite-host cell interaction, using the CHO cell adherence assay. Amebic cells were pretreated with preimmune or test rabbit anti-LecA antiserum, mixed with CHO cells, and the ability of amebae to adhere to CHO cells was quantitated. Preincubation with antiserum

significantly decreased adherence from $24.7 \pm 3.9\%$ to $7.3 \pm 2\%$ (at 1:10 dilution, $p < 0.005$) and from 31 ± 6 to $15.3 \pm 3.5\%$ (at 1:100 dilution, $p < 0.005$) in comparison to the sham serum (Fig. 4). These results showed that anti-LecA antiserum recognized the amebic surface lectin and interfered with the ability of the parasite to bind CHO cells. Addition of galactose blocked the binding of trophozoites to CHO cells as expected.

3.3. Immunization with LecA confers protection

CBA/J mice were immunized with adjuvant EM014 alone (control group), EM014 + LecA or EM014 + his tagged LecA as described under the methods section. Mice were challenged with 2×10^6 log phase trophozoites at week 7. On week 8, amebic infection in the ceca from sacrificed mice was evaluated by ELISA and by culture in TYI-S-33 media. Vaccination with LecA resulted in a significant decrease in amebic load. The mean OD value for the vaccinated mice was 1.17 ± 0.5 whereas that for the control mice was 5.4 ± 1.1 ($p < 0.05$, Fig. 5A). 60% mice in the LecA vaccinated group showed $\pm$ values below the baseline (OD 0.05) as compared to only 20% in the control group, indicating very low levels of antigen in the vaccinated mice. Culture results reflected the ELISA observations as only 20% were infected in the LecA-immunized group compared to 60% mice in the control group ($p < 0.05$, Fig. 5B). The protection, measured by the standard methods of antigen load and culture positivity, were comparable between untagged and His-tagged LecA formulations.

4. Discussion

We previously showed that his-tagged LecA afforded protection in a mouse model of amebic colitis [16]. However, there were difficulties in achieving his-tagged LecA preparations of a consistent degree of purity, and there were concerns about the potential long-term effect of anti-his-tag antibodies elicited by vaccines with his-tagged protein preparations. Therefore, we developed a scalable purification process that yielded sufficient amounts of highly purified non-tagged LecA for characterization, immunogenicity, and protection studies. Our results showed that LecA was produced primarily as IB, regardless of the *E. coli* host, vector, or growth conditions tested. We initially suspected that IB resulted from disulfide linkages due to the unusually high cysteine content in this cysteine-rich portion of the heavy chain subunit, but were unable to prevent their formation with reducing/denaturing agents. By using guanidine-HCl and arginine in a redox environment with reduced and oxidized glutathione in the refolding process, we were able to solubilize and refold the protein in a reproducible fashion. Refolding may be optimized by using low protein concentrations, but for the purposes of our scalable purification process, this was not optimal because of the amount of protein purified. L-arginine has previously been shown to increase repulsive rather than attraction interaction between proteins [18]. This effect, in turn, improves the refolding process by increasing the yield of soluble protein. Thus, L-arginine incorporated into the refolding step for LecA made our process more scalable. Although the formation of IB made purification more challenging, it allowed us to remove most of the contaminating lysate proteins by washing and collection by centrifugation of IB.

Size exclusion enabled us to obtain LecA preparations of consistent purity with reduced levels of endotoxin. Levels of <20 EU/mL have been recommended for recombinant subunit vaccines, although this concentration still is unclear since endotoxin levels often are specific to a licensed vaccine, and generalized guidance statements only refer to the U.S. Food and Drug Administration endotoxin limit of 5 EU/kg/h for intravenous drugs [15,44]. For our studies, we can now produce LecA with more accurately defined endotoxin levels that fall under guideline levels and which is protective. This is important since, as noted by Brito and Singh [6], immunogenicity may decrease as endotoxin is removed during a purification process.

By incorporating a gentle L-arginine refolding process, we were able to improve folding and decrease aggregation, as demonstrated by immunoassay analysis and comparison with native lectin and his-tagged lectin, both of which are protective in animal models [16,19]. Linear regression analysis showed that refolded LecA exhibited slope values higher than those observed with his-tagged LecA, and that when heat-treated, LecA was no longer reactive. These findings indicated proper recognition and binding of antibodies to the protein, further supporting proper refolding. The monoclonal antibodies used in our evaluation have been shown previously to bind to conformationally dependent epitopes [25]. All bind effectively to his-tagged LecA and nontagged LecA in a manner consistent with that observed with native lectin, indicating proper refolding of LecA. These observations, and the demonstration that (i) anti-LecA antiserum inhibited the binding of CHO cells to trophozoites, (ii) immunization with LecA reduced antigen load in a mouse model of amebic colitis, and (iii) immunization with LecA protected mice from infection, showed that nontagged LecA was as effective as his-tagged LecA and native lectin, and that key antigenic properties of lectin were preserved [16,19].

Over 90% of human amebiasis is due to amebic colitis, and not the liver abscess for which animal models have long been available. In this study, we used the recently developed CBA mouse model of amebic colitis in which mice are infected by injection of amebic trophozoites into the cecum. Chronic *E. histolytica* infection with ulcerative colitis develops in 60–90% of infected C3H mice and reproducibly 80% of CBA mice; histopathologically the lesions in the mouse colon closely resemble human amebic colitis. Without intervention, *E. histolytica* infection and amebic colitis persist for months. We now have demonstrated that LecA which is not his tagged avoids potential problems that may occur with his tagged vaccines, that LecA can now be produced in a scalable manner, and importantly, that LecA prevents amebic infection and colitis in a highly relevant mouse model of amebiasis.

As a purified recombinant antigen, LecA requires the use of an adjuvant formulation in order to enhance immunogenicity and protective efficacy. EM014 consists of GLA-SE (a synthetic TLR4 ligand formulated in a stable oil-in-water emulsion) combined with the synthetic TLR9 ligand CpG 1826. EM014 induces a Th1-type immune response, resulting in the highest levels of splenocyte IFN- γ production and protective efficacy against amebiasis when compared to a range of other adjuvant formulations, including Freund's adjuvant [16,44]. EM014 represents an adjuvant formulation with a potential path forward as a component of a vaccine product, since both GLA-SE and CpGs have been evaluated in completed and ongoing clinical trials with recombinant vaccine antigens (www.clinicaltrials.gov).

The high molecular mass of LecA, more than twice the predicted size or size determined by SDS-PAGE, supported the circular dichroism results showing that at the molecular level, about half of the molecule exists primarily in an unfolded state. Therefore, one possibility is that LecA exists with a large Stokes radius accompanied by a high percentage of random coil structure and β -sheet structure. Alternatively, it is possible that in the native configuration, LecA exists as a dimer, which would also explain the high mass. Titration with Ellman's reagent detected all predicted 31 sulfhydryl groups in denatured LecA even though none was accessible in nondenatured LecA. Thus, the odd number of sulfhydryl groups may indicate that at least one of the sulfhydryl groups is accessible in the native molecule, and participates in dimer formation. Size exclusion profiles did not change in the presence of dithiothreitol, suggesting that if such a disulfide linkage exists, it is very stable. In any event, our data support the idea that sulfhydryl groups in LecA are engaged in intramolecular interactions. The purified peptide is stable since we did not observe any further unfolding, as determined by circular dichroism spectra or loss of immunoreactivity following storage at 18 months or after lyophilization.

The process that we developed yielded >20 mg of highly purified LecA protein per liter of culture, the purified preparations have acceptably low endotoxin levels, the LecA elicits a protective response in mice, and the purification process is scalable for cGMP. The availability of highly purified LecA will allow us to proceed with antigen dose/potency studies, long term stability testing, further analysis of peptide secondary structure, and development of additional acceptance criteria for quality control. The Gal/GalNAc lectin is the best studied antigen of *E. histolytica*, and currently LecA is the most logical choice as a vaccine candidate. We now have a process to produce sufficient amounts of a properly folded recombinant peptide for new detailed studies on this antigen and its consideration as a vaccine. This is a significant step toward generating a human amebic vaccine.

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Fig. 1.

Schematic representation of the heavy chain subunit of the Gal/GalNAc lectin of *E. histolytica*. The Gal/GalNAc lectin is a 260 kDa heterotrimer of disulfide-linked heavy (Hgl) and light (Lgl) subunits non-covalently associated with an intermediate subunit (IgI). All 3 subunits are encoded by gene families. The sequence of the hgl genes is nearly completely conserved in isolates of *E. histolytica* from different continents. LecA, shown in gray, is located within the heavy chain subunit and is a vaccine candidate because (i) it contains the carbohydrate recognition domain (CRD), a cysteine-rich region recognized by adherence-inhibitory MAb that when expressed in *E. coli*, binds to Gal/GalNAc and (ii) active immunization against LecA is protective in animal models.

Fig. 2. Superdex 200 size exclusion profiles of Lot #s 071111RSW (A), 071111LAB (B), and 071411LAB (C) Stage 2 LecA preparations. The A₂₈₀ (blue) and A₂₅₄ (red) are presented on the left and right y-axis, respectively, in each panel and the elution volume is presented on the x-axis. Fractions that were included in the Stage 3 Pool of each lot are shown in each chromatogram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3.

Electrophoretic analysis of LecA preparations. The left lane in each pattern contained the same molecular markers with the designated molecular mass shown on the left. Lanes 1–3 represented Lot #s 071111RSW, 071111LAB, and 071411LAB, respectively. For SDS-PAGE analysis, each lane contained 10 μ g protein. For silver stain and immunoblotting with 7F4 MAb and 3F4 MAb, Lanes 1–3 each contained 2.5 μ g protein. SDS-PAGE for silver staining was performed in 0.01 M MES buffer, pH 7.5, accounting for the slightly slower migration of the proteins. Native gel analysis was performed using 10 μ g protein per lane.

Fig. 4.

LecA serum reduces the ability of *E. histolytica* trophozoites to adhere to CHO cells. Pretreated or control *E. histolytica* trophozoites were incubated on ice for 90 min with CHO cells at a 1:20 ratio and vortexed briefly before counting under a microscope. Parasites with at least 3 adherent CHO cells were considered positive (rosette). Control samples did not contain any serum, and galactose denotes sample tubes with galactose added to the final concentration of 50 mM. Each sample was done in triplicate and at least 100 trophozoites were counted in each tube. (* $p < 0.005$; ** $p < 0.05$).

Fig. 5.

LecA mediated protection. CBA mice were immunized subcutaneously at weeks 0, 2 and 4 using untagged (LecA) or his-tagged (His-LecA) antigen formulated with EM014 adjuvant. Control (EM014 only) and vaccinated mice were challenged three weeks after the final immunization and sacrificed one week post challenge. Cecal contents were analyzed for (A) antigen load using *E. histolytica* II ELISA kit (TechLab) and (B) culture positivity by growth in TYI-S-33 medium. (* $p < 0.05$; ** $p < 0.005$).

Table 1

Protein purification and analysis of purified LecA preparations. Assays were performed in duplicate and represent average values. The total protein yield for Lot #071111LAB was lower than the other two lots because only half of the starting material was processed. The extrapolated yield for this particular lot was comparable to that observed with the other two lots.

Lot #	Total protein ^a (mg)	Endotoxin ^b (EU/μg)	DNA ^c (ng/mg)	RNA ^c (ng/mg)	Analytical sensitivity ^d (ng detected)	Linear regression analysis ^d
071111RSW	27.0	0.01	51	<20	0.14	0.71
071111LAB	12.9	0.01	82	<20	0.11	0.77
071411LAB	24.7	0.02	61	<20	0.31	0.74

^aProtein was determined using the Thermo Scientific/Pierce Modified Lowry Assay (Rockford, IL).

^bEndotoxin levels were determined using the FDA-licensed Endosafe PTS™ cartridges and reader from Charles River Laboratories.

^cDouble-stranded (ds) DNA and RNA were quantified using the Qubit™ Fluorimeter and Life Technologies kits.

^dAnalytical sensitivity and linear regression analysis was determined using a modified quantitative *E. HISTOLYTICA II* kit.