Prevention of intestinal amebiasis by vaccination with the Entamoeba histolytica Gal/GalNac lectin

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Abstract

Prevention of intestinal infection by Entamoeba histolytica would block both invasive disease and parasite transmission. The amebic Gal/GalNAc lectin mediates parasite adherence to the colonic surface and fecal anti-lectin IgA is associated with protection from intestinal reinfection in children. We tested if vaccination with the E. histolytica Gal/GalNAc lectin could prevent cecal infection in a C3H mouse model of amebic colitis. Two trials using native lectin purified from the parasite and two trials using a 60 kDa recombinant fragment (“LecA”) were performed with a combined intranasal and intraperitoneal immunization regimen using cholera toxin and Freund’s adjuvants, respectively. Two weeks after immunization mice were challenged intracecally with trophozoites, and 4–12 weeks after challenge mice were sacrificed for histopathologic evaluation of infection. Vaccination prevented intestinal infection with efficacies of 84 and 100% in the two native lectin trials and 91 and 34% in the two LecA trials. Mice with detectable pre-challenge fecal anti-lectin IgA responses were significantly more resistant to infection than mice without fecal anti-lectin IgA responses. These results show for the first time that immunization with the Gal/GalNAc lectin can prevent intestinal amebiasis in mice and suggest a protective role for fecal anti-lectin IgA in vivo.

Keywords: Entamoeba histolytica; IgA; Mouse

1. Introduction

Amebic colitis and liver abscess result from infection with the parasite Entamoeba histolytica. The WHO estimates that amebiasis is the third leading parasitic cause of death [1]. Recent prospective data has demonstrated a 2-year incidence of amebic colitis of 4% in Bangladeshi children and an annual incidence of amebic liver abscesses of over 21 per 100,000 Vietnamese adults [2,3]. Prevention of intestinal infection through vaccination would theoretically block not only these invasive sequelae but also parasite colonization [4]. Amebic trophozoites adhere in vitro to epithelial cells and colonic mucins via a cell surface lectin that recognizes galactose and N-acetylgalactosamine (Gal/GalNAc) [5]. The Gal/GalNAc lectin is a 260 kDa heterodimer of disulfide-linked heavy and light subunits that are noncovalently associated with an intermediate 150 kDa subunit [6]. Vaccination with parasite-purified Gal/GalNAc lectin [7–9] or recombinant lectin fragments [10–14] has provided protection in rodent models of amebic liver abscess, with efficacies ranging from 33 to 86%. Passive transfer of immune sera or monoclonal antibodies to lectin fragments has demonstrated that this protection is mediated at least in part by anti-lectin antibodies [8,10,15].

A correlation has been observed between the presence of fecal IgA antibody to native Gal/GalNAc lectin or the lectin carbohydrate recognition domain (CRD) and protection from intestinal colonization in humans [2,16]. Secretary IgA to native lectin or lectin fragments prevents amebic adherence to colonic mucin and epithelial cells in vitro, providing a putative mechanism for this protection [17,18].

The utility of vaccination against intestinal disease has never been tested because animal models of intestinal infection have historically been unreliable. Here we utilized a C3H mouse model of amebic colitis [19,20] to test the efficacy of vaccination using both purified lectin and a recombinant fragment of the lectin heavy subunit that contains the CRD (termed “LecA”) [21]. We show for the first time that vaccination can prevent intestinal amebiasis and demon-
straté a correlation between fecal anti-lectin IgA antibody and protection.

2. Materials and methods

2.1. Mice

Three to 6-week-old female C3H/HeJ and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were maintained under specific pathogen-free conditions at the University of Virginia. All protocols were approved by the Institutional Animal Care and Use Committee.

2.2. Parasites and antigens

*E. histolytica* strain HM1:IMSS trophozoites in axenic culture were passed four times through hamster liver abscess. The parasites recovered from the liver abscess were then cultured in 0.01% erythromycin-supplemented tryptose–yeast–iron (TYI-S-33) media [22] with the bacterial flora of strain CDC:0784.

The native *E. histolytica* Gal/GalNAc lectin was purified from strain HM1:IMSS trophozoites grown under axenic conditions as described previously [5]. A large fragment of the Gal/GalNAc lectin heavy subunit spanning amino acids 578–1154 (“LecA”) was cloned into a pRSET-A vector (Invitrogen, Carlsbad, CA) with a kanamycin resistance gene and expressed in *E. coli*. The *E. coli* cells were lysed by sonication and isolated inclusion bodies were denatured in inclusion body solubilization reagent (Pierce, Rockford, IL). The LecA fragment was also cloned into pT7-7 to eliminate the polyhistidine tag; for this purification *E. coli* were lysed in CellLytic™ B II bacterial cell lysis extraction reagent (Sigma Chemical Co., St. Louis, MO) and isolated inclusion bodies were denatured in 8 M urea. Both LecA proteins were further purified by pH titration in 0.05 M sodium acetate and cation-exchange chromatography using SP Sepharose™ fast flow (Amersham Pharmacia Biotech, Piscataway, NJ), >90% pure based on SDS-PAGE analysis, and exhibited comparable specific immunoreactivity in ENTAMOEBA and *E. histolytica* II (TechLab Inc., Blacksburg, VA) ELISA kits.

2.3. Vaccinations

Mice were immunized with a combined intranasal and intraperitoneal regimen over 6–9 weeks as specified in Table 1. Intranasal immunizations used 10 μg of antigen and 1 μg of cholera toxin (Sigma) administered intranasally in 20 μl of PBS into C3H mice under isoflurane anesthesia. Intraperitoneal immunizations used 15 μg of antigen emulsified in equal volumes of either complete (CFA) or incomplete Freund’s adjuvant (IFA) (Gibco, Grand Island, NY) injected via 20 gauge syringe. Trial 1 utilized 150 μl of complete Freund’s adjuvant, the week 4 immunization of trial two utilized 100 μl of CFA, and all other i.p. immunizations utilized 150 μl of incomplete Freund’s adjuvant.

Sham-immunized mice from each trial were administered an identical regimen of PBS with adjuvant. Mice were challenged intracereally with trophozoites 2 weeks after the final immunization.

2.4. Measurement of antibody in sera and stool

Sera were obtained from orbital plexus blood on the day prior to challenge from the lectin-1 trial. Stool suspensions were obtained in the week prior to challenge for all trials. Briefly, four stool pellets were vortexed into 1.0 ml of complete protease buffer (Roche, Mannheim, Germany) at room temperature. Suspenisons were centrifuged at 900 × g at 4°C for 10 min, transferred to fresh tubes, centrifuged again at 15,800 × g at 4°C for 10 min, and the final supernatants were removed to a fresh tube containing PMSF (2 mM final concentration). Both sera and fecal preparations were stored at −20°C prior to assay.

Total fecal IgA, antigen-specific fecal IgA, and antigen-specific serum IgG to native lectin and LecA were assayed in duplicate by ELISA as described [23]. Briefly, Immulon IV HBX (Dynex Technologies, Chantilly, VA) or Costar HB type 1 (Corning, Acton, MA) microtiter plates were coated with 0.35 μg of native lectin, 0.1 μg of LecA, or 0.25 μg of unlabeled goat anti-mouse IgA (Southern Biotechnology Associates, Birmingham, AL) and incubated at 37°C for 1 h. Plates were washed six times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (Sigma) and then blocked with PBS containing 1% bovine serum albumin (Serologicals Proteins, Kankakee, IL). Plates were then washed six times with PBS–Tween and incubated with five-fold serial dilutions of serum or fecal preparations in PBS–Tween containing 1% BSA at 37°C. Starting dilutions were 1:1 for antigen-specific fecal IgA, 1:625 for antigen-specific serum IgG, and 1:625 for total fecal IgA determinations. Plates were then washed six times with PBS–Tween and incubated for 1 h at 37°C with horseradish peroxidase-conjugated goat anti-mouse IgA or IgG sec-
ordinary antibodies (Southern Biotechnology Associates) diluted 1:2500 in PBS–Tween containing 1% BSA. Plates were then washed six times with PBS–Tween and the bound antibodies were detected using 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After stopping the reaction with 1.0 M phosphoric acid, plates were read at 450 nm on an automated microplate reader. Background absorbance from PBS-only wells was subtracted from each value and the duplicate values for each sample were averaged. A positive titer was defined as the highest dilution giving an absorbance ≥ the mean + 2 S.D. for the sham-immunized mice from the same trial. An IgA titer of ≤5 was considered absent.

For total IgA determinations, a monoclonal IgA (TEPC-15, Sigma) was used as a standard, and the total amount of IgA in each sample was calculated using a polynomial standard curve. The titer of specific IgA (reciprocal log_{10} dilution) was then divided by the quantity of total IgA in the sample (μg).

2.5. CHO cell adherence assays

Chinese hamster ovary (CHO) cell adherence assays were performed as previously described [24]. Briefly, CHO cells were cultured in MEM-α medium, released by trypsinization, and suspended with trophozoites at 4 °C in M199 media supplemented with 0.5% BSA, 5.7 mM cysteine, and 25 mM HEPES. Trophozoites and CHO cells were then centrifuged together, incubated for 1 h at 4 °C, and resuspended by vortexing. Adherence was measured as the number of trophozoites having at least three adherent CHO cells and reported as percent of adherence in paired studies.

2.6. Infection of mice with E. histolytica

For intracecal challenge of mice, the hamster-challenged trophozoites were grown in 75 cm² flasks to log-phase, incubated on ice for 5 min, and sedimented (900 × g, 5 min). The pellet was resuspended and 150 μl (~1 × 10⁷ trophozoites) was injected into the cecum after laparotomy as described above the negative control was considered positive.

2.7. Histopathology of mouse ceca

Mice were sacrificed 4–12 weeks after challenge and the cecum was fixed in 10% buffered formalin phosphate or Hollande’s fixative, cut into four to six equal cross-sections, embedded in paraffin, and 4 μm slides stained with hematoxylin and eosin. Slides were coded so that the scorer was blinded as to whether tissue was from a vaccinated or sham group. Successful infection was readily determined at 100× magnification by the presence of luminal ameba and cecal wall inflammation. In infected ceca, the quantity of luminal ameba and severity of inflammation were scored and cecal thicknesses were measured as previously described [20].

2.8. Fecal antigen detection

Three fecal pellets were obtained during weeks 1 and 2 after intracecal challenge from each mouse in the lectin-1, lectin-2, and LecA-1 trials. Fecal E. histolytica antigen was assayed within 1 h of collection using the E. histolytica II stool ELISA kit (TechLab) according to the manufacturer’s instructions, whereby an optical density at 450 nm of ≥0.05 above the negative control was considered positive.

2.9. Statistical analysis

Proportions of infected and uninfected mice from the vaccine trials were analyzed using Fisher’s exact test. Group means were compared using the Student t or alternate Welch test. The vaccine efficacy was calculated as 100 × (1 − (% of vaccinated mice with infection)/(% of sham mice with infection)) as done previously [12]. All P-values were two-tailed.

3. Results

3.1. Immunogenicity of native Gal/GalNAc lectin or LecA in mice

Based on the observation that the presence of fecal anti-lectin IgA antibody correlates with protection against intestinal reinfection in humans [2,16], we targeted the immunization regimens towards an IgA response. Preliminary experiments indicated that intranasal immunization with native Gal/GalNAc lectin plus cholera toxin did not generate a measurable fecal IgA response unless mice were boosted intraperitoneally with CFA (data not shown). Therefore, vaccine regimens with both intranasal and intraperitoneal immunizations were used for the vaccine trials (Table 1). An unexpected complication of the lectin-1 regimen was a high rate of post-operative death after intracecal challenge, which we attributed to the intraperitoneal adhesions from the CFA or the bleeding of mice for sera on the day prior to challenge. Accordingly, in subsequent trials lower amounts of CFA or IFA were used and mice were not bled prior to challenge.

Vaccinated and control mice had fecal samples obtained after vaccination to quantify the fecal IgA response to both native lectin and LecA. The proportions of mice with measurable fecal IgA levels and the mean IgA titers are shown according to vaccine regimen in Table 2. Immunization with native lectin was more effective than LecA at eliciting fecal IgA antibodies that recognized either native lectin itself or LecA (39/39 lectin-immunized versus 24/24 LecA-immunized mice were fecal anti-lectin IgA positive, P < 0.0001; 24/39 lectin-immunized versus 7/24 LecA-immunized mice were fecal anti-LecA IgA positive, P = 0.02). Within the native lectin trials, the lectin-1 regimen was significantly more effective at eliciting IgA responses than the lectin-2 regimen in terms of both anti-native...
Fecal anti-Gal/GalNAc lectin IgA responses after vaccination with native lectin or LecA

<table>
<thead>
<tr>
<th>Vaccine trial</th>
<th>Fecal anti-native lectin IgA</th>
<th>Fecal anti-LecA IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of IgA-positive micec/total (%)</td>
<td>Titer (mean ± S.E.)</td>
</tr>
<tr>
<td>Lectin</td>
<td>23/23c (100)</td>
<td>0.36d ± 0.04</td>
</tr>
<tr>
<td>Lectin-2</td>
<td>16/16c (100)</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>LecA-1</td>
<td>0/12c (0)</td>
<td>n.d.</td>
</tr>
<tr>
<td>LecA-2</td>
<td>2/12c (17)</td>
<td>0.06 ± 0.04</td>
</tr>
</tbody>
</table>

n.d.: not detectable.

a Mice were considered fecal anti-native lectin IgA or anti-LecA IgA positive if sample o.d. ≥ average o.d. + 2 S.D. of sham mice for the trial.
b Titer of fecal anti-native lectin IgA and anti-LecA IgA were reported as the log10 reciprocal dilution per μg of total fecal IgA in the sample.
c P < 0.0001 for fecal anti-native lectin IgA positivity rates between lectin (39/39) and LecA (2/24) trials.
d P ≤ 0.005 for fecal anti-native lectin IgA titer and fecal anti-LecA IgA positivity rates between lectin-1 and lectin-2 trials.
e Twelve of 24 mice from the LecA-1 trial and 6/18 mice from the LecA-2 trial did not have fecal IgA measured.

3.2. Vaccination with lectin or LecA prevents intestinal amebiasis in mice

Two weeks after the final immunization, the mice were challenged intracecally with *E. histolytica* trophozoites. We have previously reported that amebic infection is either established or cleared during the first week after challenge and that established infection is thereafter chronic, marked by persistent luminal amebae and cecal inflammation [20]. Mice were sacrificed at 4–12 weeks post-challenge for histopathologic evaluation of infection and the numbers of infected mice were compared in the vaccinated and sham cohorts for each trial (Table 3). We found a significantly lower proportion of infected mice in the vaccinated versus sham cohorts in the lectin-1, lectin-2, and LecA-1 trials and an insignificantly lower proportion of infected mice in the LecA-2 trial (P = 0.03, <0.0001, 0.007, and 0.15, respectively). Vaccine efficacies were calculated to be 82, 100, 89, and 34% in the lectin-1, lectin-2, LecA-1, and LecA-2 trials, respectively. The poor efficacy of the LecA-2 trial versus the LecA-1 was not explained by differences in IgA response rates (Table 2) nor in the purity of the immunogen, although the LecA-2 antigen was expressed without a polyhistidine tag. Overall, there was significant protection with either native lectin or LecA vaccination (1/22 native

### Table 3
Prevention of intestinal amebiasis by vaccination

<table>
<thead>
<tr>
<th>Vaccine trial</th>
<th>Time of sacrifice (weeks post-challenge)</th>
<th>Sham-vaccinated infection rate (number of infected/total (%))</th>
<th>Gal/GalNAc lectin-vaccinated infection rate (number of infected/total (%))</th>
<th>P-valuea</th>
<th>Vaccine efficacyb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lectin</td>
<td>9–12</td>
<td>8/13 (62)</td>
<td>1/9 (11)</td>
<td>0.03</td>
<td>82</td>
</tr>
<tr>
<td>Lectin-2</td>
<td>5–7</td>
<td>17/21 (81)</td>
<td>0/13 (0)</td>
<td>&lt;0.0001</td>
<td>100</td>
</tr>
<tr>
<td>LecA-1</td>
<td>7</td>
<td>9/17 (53)</td>
<td>1/17 (6)</td>
<td>0.007</td>
<td>89</td>
</tr>
<tr>
<td>LecA-2</td>
<td>4</td>
<td>12/15 (80)</td>
<td>9/17 (53)</td>
<td>0.15</td>
<td>34</td>
</tr>
</tbody>
</table>

a Comparing infection rate of sham versus Gal/GalNAc lectin-vaccinated mice.
b Vaccine efficacy = 100 × (1 – (Gal/GalNAc lectin-vaccinated infection rate)/(sham-vaccinated infection rate)).
Fig. 1. Cecal pathology of Gal/GalNac lectin-vaccinated and sham-vaccinated C3H/HeJ mice challenged with E. histolytica. Shown are ceca of mice from the LecA-2 vaccine trial, all sacrificed 4 weeks after challenge. Vaccinated/infected and sham/infected ceca demonstrated similar pathologies, with abundant luminal amebae (arrowheads) ulcerating the epithelium (arrow), mucosal hyperplasia (bracket), and submucosal inflammation (*). In contrast, uninfected ceca showed normal mucosal thickness (bracket) without inflammation, with again no differences between the vaccinated/uninfected versus sham/uninfected groups. Total numbers of ceca with infected versus uninfected histopathology from the four vaccine trials are shown in parentheses. All photomicrographs are shown at 100×, H&E.

Lectin-vaccinated mice became infected versus 25/34 controls, $P < 0.0001$; 10/34 LecA-vaccinated mice became infected versus 21/32 controls, $P = 0.006$), but native lectin vaccination was more efficacious than LecA (94% versus 55%; $P = 0.01$).

The pathology of amebic colitis was blindly evaluated between the sham and native Gal/GalNAc lectin vaccinated mice that became infected after challenge. Both groups demonstrated luminal amebae, epithelial ulceration, mucosal hyperplasia, and submucosal inflammation, which contrasted with the normal-appearing uninfected ceca from sham or vaccinated mice (Fig. 1). Quantities of luminal amebae and inflammation were scored and cecal thickness was measured as described previously [20] and no significant differences were observed in infected mice from the sham versus vaccinated groups for any of the trials (data not shown). Thus while vaccination with native lectin or LecA significantly prevented the establishment of infection it had no measurable effect on the severity of disease when infection occurred. Of note, the vaccine-mediated prevention of intestinal infection occurred within days after challenge as measured by fecal E. histolytica antigen clearance, at least in the lectin-1, lectin-2, and LecA-1 trials for which fecal antigen measurements were obtained. Specifically, 33/37 (89%) and 36/37 (97%) of vaccinated-protected mice from these three trials were fecal antigen by weeks 1 and 2, respectively, suggesting that vaccine-mediated protection was not due to a delayed immune mechanism (data not shown).

Table 4
Correlation of fecal IgA response with resistance to intestinal amebiasis

<table>
<thead>
<tr>
<th>Pre-challenge fecal IgA response</th>
<th>Subsequent infection status</th>
<th>Resistance rate (number of resistant/total (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected (n)</td>
<td>Resistant/uninfected (n)</td>
</tr>
<tr>
<td>Vaccinated/fecal anti-native lectin IgA positive</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>Vaccinated/fecal anti-native lectin IgA negative</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Vaccinated/fecal anti-LecA IgA positive</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Vaccinated/fecal anti-LecA IgA negative</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>Sham vaccinated</td>
<td>46</td>
<td>20</td>
</tr>
</tbody>
</table>

*a: Data not available from seven mice in the LecA-1 trial and six mice in the LecA-2 trial from which fecal preparations were not obtained.

*b: $P = 0.03$ for resistance rates between vaccinated/fecal anti-native lectin IgA positive vs. vaccinated/fecal anti-native lectin IgA negative mice.

*c: $P = 0.006$ for resistance rates between vaccinated/fecal anti-native lectin IgA negative vs. sham-vaccinated mice.
3.3. Comparisons of vaccine protection with pre-challenge IgA titer

We compared the pre-challenge fecal IgA response with the subsequent infection status on a mouse-by-mouse basis and found a significant correlation between the presence of pre-challenge fecal anti-native lectin IgA and subsequent resistance to infection (Table 4). Specifically, 23/24 mice (96%) with detectable fecal anti-lectin IgA were resistant to infection while only 13/19 mice (68%) without fecal anti-lectin IgA were resistant to infection, \( P = 0.03 \). In contrast, the presence of fecal anti-LecA IgA antibody was not a significant marker of protection (14/17 mice (82%) with and 22/26 mice (85%) without fecal anti-LecA IgA were resistant to infection, \( P = \text{NS} \)). Finally, the 68% (13/19) resistance rate of the vaccinated/fecal anti-lectin IgA negative mice remained significantly higher than the 30% (20/66) baseline resistance rate of sham-vaccinated mice, \( P = 0.006 \), suggesting that multiple mechanisms of Gal/GalNac lectin vaccine-mediated protection may be operating, and that fecal anti-lectin IgA is just one marker of the immune state.

4. Discussion

The most important finding from this study is that vaccination can prevent intestinal amebiasis in mice. The rationale for using the Gal/GalNac lectin to vaccinate against *E. histolytica* comes from its role in adherence and virulence, including the protein’s requirement for contact-dependent killing of mammalian cells and its ability to inhibit complement-mediated lysis of the parasite [5,17,18,25,26].

We found that vaccination with either native lectin or a recombinant fragment spanning amino acids 578–1154 of the lectin heavy subunit prevented intestinal infection. The highest level of protection was observed in mice that developed a measurable fecal anti-lectin IgA response. This correlation parallels human data whereby fecal anti-lectin IgA is linked to a decreased risk of intestinal reinfection [16].

The finding that fecal anti-lectin IgA but not fecal anti-LecA IgA correlated with enhanced protection suggests that qualitatively important epitopes outside of LecA may add protection against intestinal disease. Such putative epitopes do not appear to add protection against experimental liver abscess insofar as vaccination with native lectin is no more protective than vaccination with recombinant proteins similar to LecA (heavy subunit amino acids 649–1202 and 758–1134) [7,11,12]. The mechanism by which lectin epitopes outside of LecA could add protection against intestinal amebiasis is unclear, as LecA spans all described Gal/GalNac lectin effector epitopes for neutralization of adherence [24], complement resistance [5], and cytokine release [27,28]. One possible explanation is that anti-lectin IgA antibodies outside of LecA can enhance mucus trapping of the parasite through mucus–Fc\(\alpha\) interactions [29].

The correlation between a fecal anti-lectin IgA response and protection was not absolute, and many vaccinated mice were protected without pre-challenge fecal IgA. Unless the absence of measurable IgA is simply due to technical limitations in measuring low titers of specific IgA in protease-rich fecal matter, one must invoke the presence of non-IgA mechanisms of vaccine protection (such as CD4\(^+\) T cell responses or IgG). It is noteworthy that the LecA-1 trial was 89% efficacious though 0/12 mice produced fecal anti-lectin IgA.

Our studies did not attempt to correlate proliferative and cytokine responses with vaccine protection, however it is interesting that the highly-protective native lectin trials utilized CPA, which has been shown to induce antigen-specific IFN-\(\gamma\) production in mice, a cytokine known to stimulate macrophage amebicidal activity in vitro [30,31].

Ultimately, these vaccine data provide only correlates of protection. Future work should definitively test the role of fecal anti-lectin IgA by passive transfer of monoclonal IgA antibodies, including those with epitopes outside of LecA, and the contribution of specific cytokines such as IFN-\(\gamma\) through neutralization. These studies can further instruct the selection of *E. histolytica* immunogen, the route of vaccine delivery, and the choice of human adjuvant.

Acknowledgements

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