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Oral Immunization with a Recombinant Cysteine-Rich Section of the *Entamoeba histolytica* Galactose-Inhibitable Lectin Elicits an Intestinal Secretory Immunoglobulin A Response That Has In Vitro Adherence Inhibition Activity

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The LC3-encoded 52-kDa recombinant protein includes amino acids 758 to 1134 of the 170-kDa subunit of the galactose-inhibitable lectin. Oral immunization of BALB/c mice with the LC3-encoded protein and cholera holotoxin induced an intestinal secretory immunoglobulin A (IgA) response ($P < 0.01$ compared with the control). There was a negative correlation ($P = 0.001$) between intestinal anti-LC3 IgA and serum IgA and IgG antibody responses. Intestinal secretions from immunized mice completely inhibited the galactose-specific adherence of axenic trophozoites to Chinese hamster ovary cells ($P < 0.01$).

Adherence of the *Entamoeba histolytica* trophozoites to colonic mucins and epithelial cells and subsequent cytolytic activity is dependent upon attachment via the parasite's 260-kDa galactose-inhibitable adherence lectin. Monoclonal antibodies directed against the lectin 170-kDa heavy subunit inhibit galactose-specific binding of trophozoites to colonic mucins and intestinal epithelial cells in vitro (3, 4). Recombinant LC3-encoded protein, the cysteine rich portion of the 170-kDa lectin subunit, is highly antigenic and immunogenic and is protective as a subunit vaccine with Titremax as an adjuvant in the gerbil model of amebic liver abscess (25). Similar to results with native lectin, over 95% of the sera from a group of 113 patients with amebic liver abscess tested positive for anti-LC3 serum immunoglobulin A (IgA) by enzyme-linked immunosorbent assay (ELISA) (25).

IgA plays a major role in the exclusion of microorganisms and environmental antigens from mucosal surfaces. In vitro studies with antilectin monoclonal antibodies (22) and research with humans characterizing the secretory IgA (sIgA) response (1, 5, 8, 12, 14) suggest a role for mucosal antilectin IgA in protective immunity against intestinal infection. Antilectin sIgA has been found in the saliva of patients with amebic colitis and liver abscess (2, 14). Carrero et al. (2) demonstrated that saliva from patients with amebic colitis contained antilectin sIgA. Both the saliva and purified sIgA inhibited the adherence of amebic trophozoites to MDCK cell monolayers in vitro (2).

We report that oral immunization with the recombinant LC3-encoded fusion protein with cholera toxin (CT) as an adjuvant elicits high titers of anti-LC3 intestinal sIgA antibodies that block the in vitro galactose-specific adherence of axenic *E. histolytica* trophozoites to Chinese hamster ovary (CHO) cells.

Immunization protocol. Eight-week-old female BALB/c mice (Charles River Breeding Laboratories, Inc., Willington, Mass.) were lightly anesthetized by intraperitoneal injection of 1.0 mg of ketamine prior to intragastric immunization. Hanks' balanced salt solution and sodium bicarbonate solution (8:2) were administered intragastrically 15 min before immunization to neutralize stomach acid. Mice were immunized with saline with or without 10 μg of CT (holotoxin) (List Biochemical, Campbell, Calif.) or with saline with LC3-encoded protein (0.5 or 1.0 mg) and 10 μg of CT. Intragastric immunizations were administered to mice on days 0, 7, 14, and 21; sera and intestinal secretions were collected on day 28. Results are expressed as mean optical density (OD) + standard error.

Preparation of the recombinant LC3-encoded protein and protein fragments. The LC3-encoded protein (amino acids [aa] 758 to 1134 of the lectin 170-kDa subunit) was expressed and purified as described previously (25) by utilizing the pRSET expression vectors (Invitrogen, San Diego, Calif.) in *Escherichia coli* BL21(DE3)(pLysS) (Novagen, Madison, Wis.). The protein concentration was determined by dye-binding assay (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin (BSA) as a standard. The purity was determined by migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and Coomassie blue staining.

Three segments of the LC3-encoded protein were generated as follows. The LC3 gene was split with restriction enzymes, using combinations of *Bam*HI and *Spe*I, *Bam*HI and *Hin*dII, and *Eco*RI. The three fragments were subsequently subcloned in frame into pRSET expression vectors to generate the respective recombinant proteins, BS (aa 758 to 868), BH (aa 758 to 994), and HE (aa 944 to 1134).

Collection of serum and intestinal fluid. Blood was collected via the right axillary artery following metaphane (Pitman Moore, Mundelein, Ill.) inhalation and allowed to clot for 24 h at 4°C. Serum samples were stored at −70°C until processed for ELISA. Mice were sacrificed by cervical dislocation.

Intestinal secretions were collected by using a lavage solution (25 mM NaCl, 40 mM Na_2SO_4, 10 mM KCl, 20 mM $NaHCO_3$, 48.5 mM polyethylene glycol [average molecular mass = 3,350 g], 0.05 mg of soybean trypsin inhibitor per ml, and 25 mM EDTA) modified from the method of Elson et al. (7). Following exsanguination, the peritoneal cavity was opened, and the intestine from the duodenum to just past the cecum was clamped off, surgically removed, and rinsed twice in

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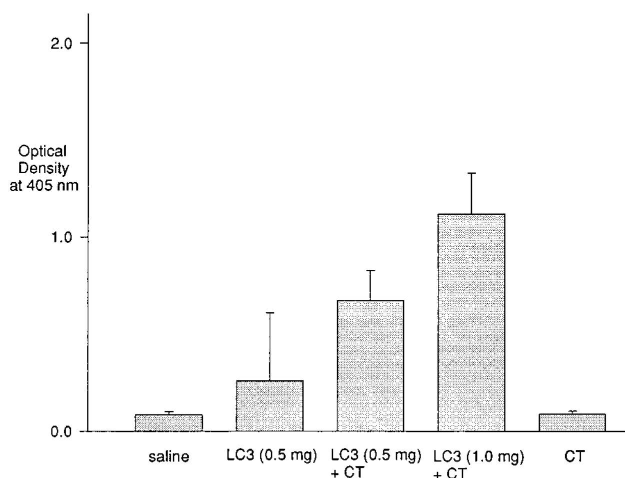

FIG. 1. Anti-LC3 intestinal sIgA responses (day 28) induced by oral immunization. A comparison of anti-LC3 IgA responses in mice given recombinant LC3-encoded protein orally with or without CT ($n = 4$) is shown. Sham-immunized mice received saline ($n = 13$) or CT alone ($n = 14$). There was no significant difference in the responses of mice given 0.5 or 1.0 mg of LC3-encoded protein with CT ($n = 13$ and 14, respectively; $P \geq 0.10$), both of which were significantly above the responses of CT or saline controls ($P \leq 0.01$). Mice immunized with LC3-encoded protein (0.5 mg) without CT as an adjuvant had significantly lower levels of intestinal anti-LC3 IgA than did mice immunized with 0.5 or 1.0 mg of LC3-encoded protein with CT ($P \leq 0.05$).

40 ml of phosphate-buffered saline to remove any blood. The intestine was opened longitudinally, cut into 3.0-cm strips, and incubated in 2.0 ml of lavage solution for 10 min at room temperature. The tissue was gently homogenized in a Dounce homogenizer with a loosely fitting pestle. The homogenized sample was placed in a 15-ml centrifuge tube, and the homogenizer and pestle were rinsed with an additional 2.0 ml of lavage solution. The rinse was combined with the homogenized sample in the centrifuge tube, and the intestinal contents were clarified by centrifugation for 10 min at 1,000 × *g*. The supernatant was transferred to microcentrifuge tubes with 0.01 volume of 100 mM phenylmethylsulfonyl fluoride (U.S. Biochemicals, Cleveland, Ohio). The samples were centrifuged for 15 min at 13,000 × *g*, the supernatant was removed, and an additional 0.01 volume of 100 mM phenylmethylsulfonyl fluoride–1% sodium azide solution was added. The clarified gut contents were placed on ice, and 0.05 volume of 3.5% globulin-free BSA solution was added. The processed intestinal contents were frozen at −70°C until studied.

Antibody analysis. Intestinal samples were assayed by ELISA for anti-LC3 IgA, and serum samples were assayed for anti-LC3 IgA and IgG. The intestinal and serum samples were tested by using 2- and 10-fold dilutions. All samples were diluted in TST buffer (30 mM Tris, 95 mM NaCl, 0.05% Tween 20, 0.002% NaN_3) with 1% BSA, pH 7.4. Each dilution was simultaneously assayed in duplicate. Results are expressed as mean OD values after correction for nonspecific binding.

High-protein-binding 96-well ELISA plates (Costar, Cambridge, Mass.) were coated overnight at 4°C with 0.2 μg of recombinant LC3-encoded fusion protein in 100 μl of coating buffer (50 mM Tris, 140 mM NaCl, 0.002% NaN_3 [pH 9.8]) per well. The plates were washed twice with wash buffer (0.05% Tween 20, 150 mM NaCl, 0.002% NaN_3), and 200 μl of 1% BSA–TST per well was added and left for 2 h at room temperature to block sites not bound by the LC3-encoded protein. The plates were washed twice, and 100 μl of sample was incubated overnight at 4°C. After incubation, the plates were washed extensively, and 100 μl of a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgA or IgG (Southern Biotech, Birmingham, Ala.) in 1% BSA–TST was added to each well. The plates were incubated for 2 h at room temperature, and this was followed by another set of five washes.

p-Nitrophenyl phosphate (Sigma, St. Louis, Mo.) (1 mg/ml in glycine buffer [50 mM glycine, 1 mM $MgCl_2$, pH 9.6]) was used as the substrate for alkaline phosphatase. OD readings at 405 nm were obtained 30 and 60 min after addition of the alkaline phosphatase substrate.

Culture of *E. histolytica* and CHO cells. *E. histolytica* HM1: IMSS was cultivated axenically in TYI-S-33 medium (6) with penicillin (100 U/ml) and streptomycin (100 μg/ml). CHO cells (American Type Culture Collection, Rockville, Md.) were grown in Ham F-12 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Effect of intestinal secretions on amebic adherence to CHO cells. Adherence was determined by a rosette assay as previously described (23). Amebae (10^5/ml) were incubated at 4°C for 30 min in 100% supplemented M199 medium (GIBCO) containing 5.7 mM cysteine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) buffer, and 1% BSA (Sigma) with or without a 1:5 dilution of intestinal secretions (dialyzed against unsupplemented M199 medium). Amebae (10^4) were washed twice with chilled supplemented M199 medium prior to suspension with CHO cells (2×10^5) in 1.0 ml of supplemented M199 medium. The samples were centrifuged at

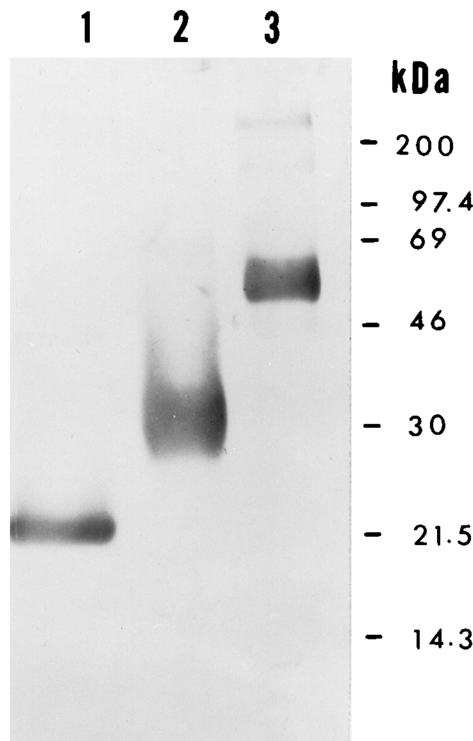

FIG. 2. Immunoblots of IgA in mouse intestinal secretions with fragments of the recombinant LC3-encoded protein. Fusion proteins resulting from expression of a restriction enzyme digest of LC3 DNA and the LC3-encoded protein were separated by SDS-PAGE, and immunoblots with mouse sIgA were performed. Lane 1, BS fragment (aa 758 to 868); lane 2, BH fragment (aa 758 to 994); lane 3, entire recombinant LC3-encoded protein (aa 758 to 1134). Data for the HE fragment (aa 944 to 1134) are not shown. Following immunization with the LC3-encoded protein, mouse intestinal IgA recognized all protein fragments.

TABLE 1. Inhibition of amebic galactose-specific adherence to CHO cells by mouse immune intestinal secretions

Sample	% Adherence[a] to CHO cells	n[b]
Medium control	68.1 ± 3.5	14
Galactose (2%) control	12.4 ± 3.1	14
Intestinal secretions from:		
Sham-immunized mice	42.4 ± 3.2	20
LC3-immunized mice	11.5 ± 2.6[c]	20

[a] Determined by rosette formation after exposure of amebae to intestinal secretions at 4°C and expressed as mean ± standard error.

[b] n = number of adherence assays performed. Intestinal secretions were pooled from studies of six mice in each group.

[c] $P \leq 0.0001$ compared with results for secretions from sham-immunized mice.

150 × *g* for 5 min and incubated at 4°C for 1 h. After incubation, 0.8 ml of the supernatant was removed, the tube was vortexed for 5 s, and the percentage of amebae with three or more adherent CHO cells was determined. For each assay, controls with and without galactose (20 μg/ml) were included.

Statistics. All comparisons of significance were made by paired or unpaired Student's *t* tests as appropriate.

Anti-LC3 IgA in intestinal samples. Mice received at 7-day intervals four intragastric immunizations with saline, saline and CT, 0.5 mg of LC3-encoded protein and CT, or 1.0 mg of LC3-encoded protein and CT. Figure 1 illustrates the average mean OD determined by ELISA for anti-LC3 IgA present in undiluted intestinal preparations 7 days after the last immunization. The combination of the LC3-encoded protein and CT induced an intestinal anti-LC3 IgA response significantly greater than that induced by antigen or CT alone ($P \leq 0.01$) (Fig. 1). The mean sIgA response elicited by 0.5 or 1.0 mg of the LC3-encoded protein was comparable ($P \geq 0.10$). Inclusion of CT as an adjuvant was crucial for induction of an IgA response, as the LC3-encoded protein alone did not induce significant levels of intestinal anti-LC3 IgA ($P > 0.05$) (Fig. 1). Mice orally immunized only two or three times had intestinal and serum anti-LC3 IgA levels significantly lower ($P < 0.05$) than those obtained after four intragastric immunizations (data not shown). Serum anti-LC3 IgA responses were also observed only when the CT adjuvant (at a 1:500 dilution) was utilized (ODs of 0.196 ± 0.19 and 0.106 ± 0.13 with 0.5 and 1.0 mg of LC3-encoded protein, respectively) ($P < 0.01$ compared with CT and saline groups). ELISAs for serum anti-LC3 IgG gave ODs of 1.81 ± 0.75 and 1.48 ± 0.90 (at a 1:500 dilution) for 0.5 and 1.0 mg, respectively, with CT ($P < 0.01$ compared with CT and saline controls).

Immunoblots of intestinal secretions with LC3-encoded fragments BH (aa 758 to 944), BS (aa 758 to 868), and HE (aa 944 to 1134) were all positive, indicating that the epitopes recognized by the sIgA antibodies were distributed throughout the protein (Fig. 2). Intestinal IgA also recognized purified native 260-kDa lectin as determined by ELISA (average OD = 0.46). Of interest was a negative correlation between intestinal IgA and both serum IgA ($r = 0.582$, $P = 0.001$) and serum IgG ($r = 0.622$, $P = 0.023$), indicating that the highest intestinal IgA responses correlated with the lowest systemic antibody responses.

Inhibition of amebic galactose-specific adherence to CHO cells by murine intestinal secretions. Intestinal secretions were evaluated for their ability to block the galactose-specific adherence of axenic trophozoites to CHO cells. Intestinal secretions (1:5 dilution) from control mice immunized with CT alone resulted in a 38% reduction in amebic adherence to CHO cells (Table 1), which we attribute to the adherence-inhibitory effects of intestinal mucins (3, 15). However, intestinal secretions from LC3-immunized mice (Table 1) completely inhibited the galactose-specific adherence of trophozoites to CHO cells ($P \leq 0.0001$).

One strategy for vaccine-mediated protection against enteric pathogens is to induce a blocking intestinal mucosal sIgA immune response (9, 21). CT is a potent adjuvant for mucosal IgA responses and promotes long-term immunologic memory in unrelated proteins in the gut mucosa (13, 20, 26). The holotoxin form of CT was used because mice require the A subunit to elicit an adjuvant effect (18, 19, 26). However, in humans the nontoxic B subunit is sufficient for an effective adjuvant (10, 11, 17).

High levels of antigen exposure without CT in the intestine can cause a state of systemic unresponsiveness while eliciting an intestinal IgA response (11, 16, 24). We found that increasing levels of intestinal IgA correlated with decreasing levels of systemic anti-LC3 immunoglobulins. A larger inoculum of LC3-encoded protein may enhance the mucosal sIgA response in the intestine and suppress the systemic and intestinal IgG responses.

Mucosal secretions from mice immunized with recombinant LC3-encoded protein completely inhibited the galactose-specific adherence of *E. histolytica* trophozoites to CHO cells. This indicates a direct role for mucosal anti-LC3 IgA in inhibition of the lectin-mediated attachment of the parasite to target CHO cells. The modest inhibition of adherence by control intestinal preparations is most likely the result of the mucins present. Colonic mucins from rats and humans are rich in oligosaccharides that contain galactose and *N*-acetyl-D-galactosamine. Purified mucins specifically bind to the parasite's surface lectin in vitro and have an inhibitory effect on amebic adherence to CHO and rat colonic epithelial cells (3, 4).

In summary, we demonstrated that intragastric immunization of BALB/c mice with the recombinant LC3-encoded protein and CT was effective in inducing an intestinal sIgA response to the recombinant and native forms of the antigen. Intestinal preparations from these mice completely inhibited the galactose-specific adherence of trophozoites to CHO cells in vitro. This study suggests a possible role for the recombinant LC3 protein as an oral subunit vaccine to elicit an effective intestinal mucosal immune response against this important worldwide enteric parasite.

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