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Protection of Gerbils from Amebic Liver Abscess by Immunization with the Galactose-Specific Adherence Lectin of *Entamoeba histolytica*

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No protective antigens from *Entamoeba histolytica* have been previously defined. We tested the ability of the galactose-specific adherence lectin of *E. histolytica* to elicit a protective immune response in conjunction with Freund's incomplete and complete adjuvants. The gerbil (*Meriones unguiculatus*) model of an experimental amebic liver abscess was used. Gerbils were immunized intraperitoneally or subcutaneously with 10 μg of the affinity-purified lectin in complete Freund's adjuvant and then at 2 and 4 weeks with 10 μg of the lectin in incomplete Freund's adjuvant. All of the immunized animals developed antilectin antibody titers of greater than 1/1,024 as measured by a radioimmunoassay. The gerbil antilectin antibodies were shown by Western immunoblotting to be directed to the heavy subunit but not the light subunit of the lectin. Immune gerbil sera inhibited amebic adherence by 100% at a 1/10 dilution. Immune and control gerbils were challenged at 6 weeks by the intrahepatic injection of 5 × 10^5 *E. histolytica* trophozoites. Four independent trials demonstrated complete protection from amebic liver abscess formation in 67% of lectin-immunized gerbils. Unexpectedly, liver abscess weights were significantly higher in the gerbils that failed to become immune than in the control animals. Our results demonstrate that the galactose lectin is a protective antigen and provide an immune-animal model to study the mechanisms of protection and potential disease exacerbation conferred by the antilectin immune response.

Amebiasis is a frequently invasive parasitic infection of the colon caused by the protozoan *Entamoeba histolytica*. Despite the existence of effective antiamebic chemotherapy, amebiasis is a leading parasitic cause of death (25). Improvements in sanitation and water supplies in developing countries are the only means presently available to prevent infection. Recent studies indicate that the production of an effective vaccine may be feasible. Protective immunity in animal models of amebic liver abscesses has been achieved by immunization with crude or high-molecular-weight fractions of amebic proteins (5, 8, 21, 22, 24). Humoral and amebicidal cell-mediated immune responses have been documented in patients recovering from invasive *E. histolytica* infections (7, 17), and evidence suggests that the recurrence of invasive amebiasis after a cure of liver abscess or colitis is unusual (3, 20).

The galactose-specific lectin has been purified from a pathogenic strain of *E. histolytica* by monoclonal antibody affinity chromatography (13, 14). This lectin mediated adherence to human colonic mucins and epithelial cells. Its inhibition with galactose prevents contact-dependent killing of target cells (2, 16). The purified lectin is a heterodimer of one 170-kDa heavy subunit linked by disulfide bonds to one 35-kDa light subunit. The 170-kDa subunit is an antigenically conserved and immunodominant antigen, whereas humoral immune responses to the 35-kDa subunit have not been detected (12, 13, 15). Because of the importance of this lectin for in vitro adherence of and cytolysis by *E. histolytica* and because of its prominent recognition by the immune system of humans who have recovered from invasive amebiasis, we have tested its potential as a protective antigen. In our study, gerbils were directly immunized with the affinity-purified lectin prior to intrahepatic challenge with *E. histolytica*. The results of repeated trials showed that an adherence-inhibitory antilectin antibody response developed in the immunized animals, with complete protection from liver abscesses being observed in 67% of the lectin-immunized animals.

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MATERIALS AND METHODS

Gerbils. Adult male inbred gerbils (*Meriones unguiculatus*), aged 7 to 9 weeks, were obtained from Tumblebrook Farms, Inc., West Brookfield, Mass. Gerbils were given food and water ad libitum and were cared for in plastic cages with hardwood chips for bedding in accordance with National Institutes of Health guidelines.

Amebae. *E. histolytica* HM1-IMSS, recently passaged in gerbil livers, was grown in axenic cultures in medium TYI-S-33 (trypticase, yeast extract, iron, and serum) with 100 U of penicillin and 100 μg of streptomycin sulfate per ml at 37°C in glass tubes (14). Trophozoites were harvested during log-phase growth after 48 to 72 h of subculturing by chilling the cultures on ice and sedimenting the amebae at 150 × *g* for 5 min.

Purification of the amebic adherence lectin by monoclonal antibody affinity chromatography. Amebic trophozoites harvested from a 72-h culture were preincubated on ice with a 1/1,000 dilution of diisopropylfluorophosphate (Sigma) before solubilization in 150 mM NaCl–50 mM Tris (pH 8.3)–0.5% Nonidet P-40 (Sigma)–5 mM EDTA (Sigma)–2 mM phenylmethylsulfonyl fluoride. The solubilized amebae were centrifuged in a Microfuge for 10 min, and the supernatant was applied at 4°C to a monoclonal antibody affinity column

consisting of 2 mg each of protein A-purified antilectin monoclonal antibodies H8-5, 7F-4, 5B-8, 3F-4, and 6D-2 immobilized on 1 to 2 ml of Affi-Gel 10 beads (Bio-Rad). The supernatant was recirculated through the column with a peristaltic pump overnight, and the column was extensively washed with the solubilization buffer described above, first with and then without Nonidet P-40. The bound amebic lectin was eluted with 0.2 N acetic acid (pH 2.5), immediately frozen, and lyophilized (13). Typical yields were 300 μg of affinity-purified lectin from 200 mg of solubilized amebic protein. The affinity-purified lectin used for immunization was at least 77% pure, as judged by laser densitometry of Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels, and was 350-fold purified from total amebic proteins, as determined by a radioimmunoassay (13).

Immunization of gerbils with the galactose-specific lectin. Adult male gerbils were immunized by intraperitoneal or subcutaneous injection of 10 μg of the affinity-purified lectin emulsified in complete Freund's adjuvant (GIBCO, Grand Island, N.Y.) and boosted with 10 μg of the lectin in incomplete Freund's adjuvant at 2 and 4 weeks. Control gerbils were sham immunized with Freund's complete and incomplete adjuvants alone. Prechallenge gerbil sera were collected at 5 weeks after the initial immunization by cardiac puncture.

Determination of levels of antilectin antibodies in serum. Polyvinyl chloride microtiter plates (Dynatech, Alexandria, Va.) were coated with 0.1 μg of affinity-purified lectin in 0.1 M bicarbonate buffer (pH 9.6) per well overnight at 4°C, and residual binding sites were blocked with 0.1 ml of 1% bovine serum albumin–phosphate-buffered saline–0.05% Tween for 1 h at room temperature. Each well was incubated with 0.1 ml of gerbil serum serially diluted in phosphate-buffered saline–Tween for 2 h, washed five times with phosphate-buffered saline–Tween, and incubated with 10^5 cpm of ^{125}I-labeled rabbit antigerbil antiserum (a kind gift from James Kazura, Case Western Reserve University) for 4 h. After being washed again, the plates were dried, and individual wells were counted in a gamma counter (Micromedic 4/200; Rohm & Haas Co., Horsham, Pa.). All assays were carried out in duplicate.

Adherence of *E. histolytica* trophozoites to CHO cells. The measurement of *E. histolytica* trophozoite adherence to CHO cells was performed as previously described (16). In brief, trophozoites (10^4) and CHO cells (2×10^5) were suspended together at 4°C in M199 medium (GIBCO) containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 6.8) (Sigma), 5.7 mM cysteine, 0.5% bovine serum albumin, and 10% heat-inactivated adult bovine serum (M199s). The trophozoites and CHO cells were centrifuged together at 150 × *g* for 5 min and incubated at 4°C for 2 h. Adherence was measured as the number of amebae having at least three adherent CHO cells upon vortex resuspension of the cellular pellet, with at least 50 amebae being counted per tube. Adherence was expressed as the percent adherence in paired studies performed with control medium. Galactose was obtained from Pfanstiehl Laboratories, Waukegan, Ill. The effect of antibodies on CHO cell adherence was measured by preincubating amebae on ice with pooled sera from control and immunized gerbils in M199s for 60 min before measuring CHO cell adherence.

SDS-PAGE and Western immunoblotting. Trophozoites were incubated with a 1/1,000 dilution of diisopropylfluorophosphate on ice and solubilized in 50 mM Tris–150 mM NaCl containing 0.5% Nonidet P-40, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. Solubilized amebae (10^4 per lane) and purified lectin (10 μg per lane) were subjected to SDS-PAGE with 7 to 10% acrylamide running gels. Molecular weight determinations were made with high-molecular-weight standards from Bio-Rad Laboratories. All samples were boiled in 4% SDS–10% β-mercaptoethanol prior to electrophoresis. The proteins from SDS-PAGE were electrophoretically transferred to 0.1-μm-pore-size nitrocellulose (Schleicher & Schuell) for Western blotting (23). The excess protein-binding capacity of the nitrocellulose was blocked with 5% nonfat dry milk (Richfood, Richmond, Va.) in 50 mM Tris–200 mM NaCl (pH 7.5) for 60 min. The nitrocellulose was incubated overnight at 4°C with gerbil serum, washed extensively, developed with a 1/1,000 dilution of rabbit antigerbil antiserum and with ^{125}I-labeled protein A (New England Nuclear), and exposed to Kodak X-Omat AR film for 4 to 12 h.

Experimental amebic liver abscesses in gerbils. Amebic liver abscesses were induced by direct inoculation of 5×10^5 axenic *E. histolytica* trophozoites by the method of Chadee and Meerovitch (1). Gerbils were anesthetized with methoxyflurane (Penthrane; Abbott Laboratories), and a laparotomy was performed. Amebae in 0.1 ml of TY1-S-33 culture medium were inoculated into the liver by multiple injections with a 25-gauge needle. A cloned isolate of *E. histolytica* HM1-IMSS recently recovered from a gerbil liver abscess was used. Gerbils were sacrificed by cardiac puncture under penthrane anesthesia at day 14 postinoculation. Liver abscesses were aspirated under sterile conditions for microscopy and axenic cultures to document the presence of *E. histolytica* trophozoites, dissected from the liver, and weighed.

RESULTS

Antigenicity of the purified lectin. The monoclonal antibody affinity chromatography-purified galactose-specific lectin consists of a 170-kDa heavy subunit and a 35-kDa light subunit. Laser densitometry of the purified lectin used in these experiments demonstrated that at least 77% of the Coomassie blue-stained protein migrated with the heavy and light subunits, with most of the rest of the stained protein consisting of proteolytic fragments of the lectin's subunits (13). Adult male gerbils were immunized subcutaneously or intraperitoneally with 10 μg of the lectin in complete Freund's adjuvant and boosted with 10 μg of the lectin in incomplete Freund's adjuvant at 2 and 4 weeks. These two immunization regimens were chosen to maximize the chances of obtaining an immune response to the limited quantities of purified antigen available from monoclonal antibody affinity chromatography. Levels of antilectin antibodies in serum at 5 weeks after initial immunization were determined by measuring gerbil antilectin antibodies bound to solid-phase adherence lectin with ^{125}I-labeled rabbit antigerbil antiserum. All of the immunized gerbils from the four trials had antilectin antibodies detectable at serum dilutions of 1/1,024 or greater; no antilectin antibodies were detected in the undiluted sera from the sham-immunized animals (data not shown). Western blots of whole trophozoite proteins probed with the sera from the immunized gerbils demonstrated that the antilectin antibodies recognized the 170-kDa but not the 35-kDa lectin subunit (Fig. 1). A similar lack of detectable antibody response to the 35-kDa subunit has been observed when mice and rabbits are immunized with the native lectin (13). The lower-molecular-weight protein bands recognized to a small extent by immune gerbil sera (Fig. 1)

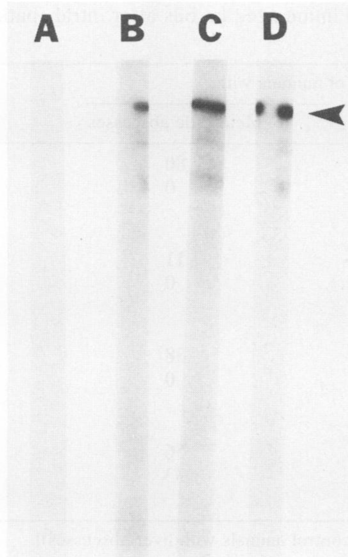

FIG. 1. Immunoblots of total amebic proteins probed with control and immune gerbil sera. Total amebic proteins electrophoresed in 10% SDS-PAGE and transferred to nitrocellulose were probed with sera from sham-immunized gerbils (lane A) and lectin-immunized gerbils (lanes B to D) prior to intrahepatic challenge with *E. histolytica*. Western blots were developed with rabbit antigerbil antiserum and ^{125}I-labeled protein A. The position of the 170-kDa heavy subunit of the lectin is indicated by the arrowhead.

most likely represented proteolytic degradation products of the 170-kDa subunit seen in purified preparations of the lectin, although an antibody response to a minor contaminant in the lectin preparation could not be ruled out.

Effect of immune sera on adherence. Amebic adherence to CHO cells can be completely inhibited by polyclonal antilectin antibodies or galactose (13, 14, 16). The effect of prechallenge sera from immunized and control gerbils on adherence to CHO cells was measured by a rosetting assay (16). Sham-immunized gerbil serum diluted from 1/10 to 1/1,000 had no effect on adherence (Table 1). Serum from gerbils immunized with the galactose-specific lectin inhibited adherence by 100% at a 1/10 dilution but enhanced adherence by 63% at a 1/1,000 dilution (Table 1). The ability of antilectin antisera at high dilutions to enhance adherence was not unexpected. Monoclonal antibodies to the lectin can enhance, inhibit, or have no effect on adherence, depending on the epitope on the lectin recognized (15). There were no significant differences observed in serum antilectin antibody titers or effects on adherence between individual animals in the immunized groups (data not shown).

TABLE 1. Effect of prechallenge sera from control and immunized gerbils on amebic adherence

Group	Serum dilution	Adherence (% of control)
Sham immunized	1/10	94 ± 3.7
	1/100	105 ± 4.1
	1/1,000	113 ± 8.7
Galactose-specific lectin immunized	1/10	0 ± 0
	1/100	47 ± 3.7
	1/1,000	163 ± 8.3

Protection studies. The gerbil model of an amebic liver abscess was used because it is one of the best-studied animal models and closely resembles the pathology of a human amebic liver abscess, with containment of the infection in a well-formed abscess. Mice have not been successfully infected with *E. histolytica* in most investigators' experiments, and inoculation of hamsters leads to a rapidly fatal disseminated infection unlike that commonly seen in humans (1). At 6 weeks after the first immunization, the gerbils were administered methoxyflurane general anesthesia and the liver was visualized by a midline abdominal incision. *E. histolytica* trophozoites (5×10^5) were directly injected into the liver with a 25-gauge needle, and the incision was closed with sutures. Two weeks after intrahepatic challenge, the gerbils were sacrificed and liver abscesses were cultured and weighed. In three separate trials with subcutaneous immunizations and one trial with intraperitoneal immunization, there was complete protection from the development of liver abscesses in the majority of the lectin-immunized animals (Table 2). When the data from the four independent trials were combined, 81% of control gerbils and 27% of immunized gerbils developed liver abscesses (two-tailed Fisher exact test, $P < 0.01$), for an overall vaccine efficacy of 67%. Extrahepatic intra-abdominal abscesses developed in 42% of control gerbils and only 10% of lectin-immunized gerbils (Table 2). Liver abscesses which developed in the lectin-immunized gerbils, however, were significantly larger than those which developed in the control gerbils. The 9 galactose-specific lectin-immunized gerbils that failed to become immune had liver abscess weights of 3.2 ± 3.7 g, and the 25 sham-immunized gerbils had liver abscess weights of 1.0 ± 0.3 g (mean ± standard deviation; $P < 0.05$).

DISCUSSION

We have demonstrated that immunization with the galactose-specific lectin of *E. histolytica* provides protection in a gerbil model of an amebic liver abscess. The identification of a specific protective antigen makes possible the future use of synthetic peptides and recombinant DNA-produced proteins for vaccine studies. Additionally, unraveling the nature of the protective immune response will be facilitated by the ability to examine cellular and humoral immune responses to a single antigen.

The production in gerbils of a polyclonal antilectin antibody response that inhibited adherence at a 1/10 dilution but enhanced adherence at a 1/1,000 dilution complicates attempts to understand the role of the antibody in protection. Murine monoclonal antibodies that bind to lectin heavy-subunit epitopes 1 and 2 have been shown to enhance adherence by directly activating the lectin's galactose-binding activity. Immune sera from patients with amebic liver abscesses also enhance or inhibit adherence, depending on the individual and the concentration of serum tested (15). The relative concentrations of adherence-inhibitory and adherence-enhancing antibodies at the site of infection may determine whether antilectin antibodies offer protection by blocking adherence to host cells. We are attempting to develop a model of colonic infection in which secretory antilectin immunoglobulin A antibody could play an important role in limiting amebic adherence and invasion.

Cell-mediated immunity may be the predominant mechanism of protection in this intrahepatic model. There were no differences in the antilectin antibody titers or adherence-inhibitory effects of the antibody in animals that were and were not protected by immunization with the lectin. Other

TABLE 2. Development of liver and metastatic abscesses in control and immunized gerbils after intrahepatic challenge with *E. histolytica*

Trial	Group	No. of animals	% of animals with:		Vaccine efficacy (liver) (%)[a]
			Liver abscesses	Metastatic abscesses	
1 (subcutaneous)	Control	5	100	80	
	Galactose-specific lectin immunized	6	33	0	67
2 (subcutaneous)	Control	9	67	11	
	Galactose-specific lectin immunized	9	22	0	67
3 (subcutaneous)	Control	8	88	38	
	Galactose-specific lectin immunized	6	50	0	43
4 (intraperitoneal)	Control	9	78	56	
	Galactose-specific lectin immunized	9	11	33	86

[a] Calculated as 100 × [1 − (percentage of immunized animals with liver abscesses/percentage of control animals with liver abscesses)].

investigators have also noted a lack of correlation of antiamebic antibody titers after immunization with protection from subsequent intrahepatic challenge with *E. histolytica* (5). Splenocytes from lectin-immunized gerbils have been shown to proliferate in the presence of lectin, secrete gamma interferon and interleukin 2, and kill amebic trophozoites in vitro (19). The production of gamma interferon may be very important in the resistance to an amebic infection. Human macrophages and neutrophils killed *E. histolytica* trophozoites upon activation with gamma interferon; in the absence of gamma interferon, these effector cells were killed by the amebae (4, 18).

The vaccine efficacy of the lectin in the gerbil model of an amebic liver abscess was lower than that previously reported for crude or fractionated amebic proteins in other animal models (5, 8, 21, 22, 24). This difference could reflect a requirement for the inclusion of additional amebic antigens during immunization to obtain maximal protection; alternatively, different schedules or routes of immunization of the lectin or different adjuvants might improve its efficacy. For example, it may be important to devise a means of obtaining an immune response to the lectin's light subunit, which was lacking in the current studies. The recent cloning of the lectin heavy-subunit cDNA promises to overcome the current limitation in the amount of lectin that can be purified for testing vaccine regimens and should enable the identification of T- and B-cell epitopes on the molecule (9).

A possible immunosuppressive role of the lectin was suggested by the larger liver abscess size in the lectin-immunized gerbils that failed to become immune. The lectin light subunit is recognized by antisera specific for the phosphatidylinositol-glycan anchor of the *Trypanosoma brucei* variant surface glycoprotein (10). The presence of a phosphatidylinositol-glycan anchor on the lectin could be one explanation for its potential immunosuppressive action. Handman and Mitchell showed that the phosphatidylinositol-glycan anchored *Leishmania* lipophosphoglycan was immunoprotective only when the anchor was intact; the formation of a soluble lipophosphoglycan by anchor hydrolysis made the molecule immunosuppressive (6, 11). In addition to being a practical problem in the formation of an effective vaccine, immunosuppression may be important in the pathogenesis of natural infections with this organism. The identification of the galactose-specific lectin of *E. histolytica* as a protective and possibly also immunosuppressive antigen promises to increase our understanding of the immune response to this infection.

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