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Recognition of the Galactose- or *N*-Acetylgalactosamine-Binding Lectin of *Entamoeba histolytica* by Human Immune Sera

WILLIAM A. PETRI, JR.,[1] M. PATRICIA JOYCE,[1] JENNIFER BROMAN,[1] ROGER D. SMITH,[2] CHERYL F. MURPHY,[1] AND JONATHAN I. RAVDIN[1]*

Divisions of Clinical Pharmacology, Geographic Medicine, and Infectious Diseases, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908,[1] and Department of Pathology, University of Cincinnati School of Medicine, Cincinnati, Ohio 45267[2]

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Cure of amebic liver abscess is associated with resistance to recurrent invasive amebiasis and the development of a humoral and cell-mediated immune response. We determined whether human immune sera contain blocking antibody for the 170-kilodalton (kDa) galactose or *N*-acetylgalactosamine (Gal/GalNAc)-binding lectin of *Entamoeba histolytica*. By Western blot (immunoblot) of whole amebae subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, all eight immune sera studied here prominently recognized a 170-kDa amebic protein. Western blot of the purified Gal/GalNAc lectin with pooled human immune sera (PHIS) confirmed that the 170-kDa band was the adherence lectin. Immunoprecipitation of [^{35}S]methionine-metabolically-labeled amebae with the antilectin monoclonal antibody H8-5 and with PHIS demonstrated that the 170-kDa lectin was the major antigen recognized by PHIS. The in vitro adherence of *E. histolytica* trophozoites to CHO cells at 4°C was inhibited by prior exposure of amebae to ≥1.0% PHIS. The humoral response to the Gal/GalNAc-binding lectin of the parasite may contribute to the development of protective immunity against invasive amebiasis.

Amebiasis is prevalent worldwide and results in approximately 50 million cases of disease and up to 100,000 deaths per year (20). Recurrence of liver abscess or documented invasive colitis due to *Entamoeba histolytica* is unusual (5, 17); it is unknown whether resistance to intestinal colonization develops after invasive amebiasis. A cell-mediated immune response occurs after invasive amebic liver abscess is cured. This consists of an antigen-specific T-lymphocyte proliferation, lymphokine production, and cytotoxic T-cell activity in vitro against amebic trophozoites (16, 16a). A humoral response develops during invasive disease by *E. histolytica*, with up to 95% of sera from patients with amebic liver abscess containing antiamebic antibody, as determined by indirect hemagglutination or other serologic methods (7, 17). Although antibody titers have not been shown to correlate with the course of the disease (7), the relation of antiamebic antibody to the development of protective immunity has not been determined.

The adherence of amebae to intestinal mucus, colonic cells, and host leukocytes is required but not sufficient for pathogenesis of invasive amebiasis (4, 19). Ravdin and co-workers have demonstrated that the in vitro adherence of *E. histolytica* trophozoites to CHO cells, human leukocytes, rat and human colonic mucosa, and colonic mucins is mediated by a galactose or *N*-acetylgalactosamine (Gal/GalNAc)-inhibitable lectin on the parasite surface (12–15; K. Chadee, W. A. Petri, Jr., and J. I. Ravdin, J. Clin. Invest., in press); studies of the interaction of amebae with bacteria confirmed these findings (2).

We recently produced seven mouse monoclonal antibodies which inhibited amebic adherence to target CHO cells (15). Petri et al. (W. A. Petri, Jr., R. D. Smith, P. H. Schlesinger, C. F. Murphy, and J. I. Ravdin, J. Clin. Invest., in press) have recently isolated the 170-kilodalton (kDa) Gal/GalNAc-binding lectin from culture supernatants or detergent-solubilized amebae by affinity chromatography with Gal-terminal glycopeptides and by affinity chromatography or immunoblotting with the lectin-specific mouse monoclonal antibodies H8-5 and F14. The purpose of this study was to determine whether immune sera from patients treated for amebic liver abscess contained antibody which recognized and functionally inhibited the amebic Gal/GalNAc adherence lectin.

MATERIALS AND METHODS

Subjects. Human sera were obtained from eight subjects treated for amebic liver abscess and from eight seronegative controls (as determined by indirect hemagglutination or counter-immunoelectrophoresis), who were matched for age, sex, and geographical location. Criteria for diagnosis and specific clinical data on these patients and controls have been previously reported (16). Seven patients were treated at the Hospital General Centro Medico Nacional (Instituto Mexicano del Seguro Social), Mexico City, Mexico, and one patient was treated at the University of Virginia. In some studies, a pooled human immune serum (PHIS) from four patients or a pooled reference immune human serum (PDB M84) obtained from George Healy at the Centers for Disease Control, Atlanta, Ga., was utilized.

Maintenance and harvesting of *E. histolytica* trophozoites and CHO cells. Axenic amebae, a clone of strain HM1-IMSS (provided by L. Diamond, National Institutes of Health, Bethesda, Md.), have been in culture in our laboratory for approximately 3 years. Strain HM1-IMSS is considered one of the most virulent axenic strains available (9, 14). Amebae were grown in tissue culture medium TYI-S-33 (6) and were maintained and harvested as previously reported (11, 12, 14). Amebae used in experiments were in the logarithmic phase of growth within 72 h of subculture. CHO cells were grown in F-12 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO), peni-

* Corresponding author.

cillin (100 μg/ml), and streptomycin (100 μg/ml) and were harvested by trypsinization as described previously (11).

Preparation of *E. histolytica* protein. Amebae were pelleted at 4°C at 150 × *g* for 5 min from 10 ml of a 24-h culture containing 2 × 10^5 trophozoites per ml. The amebae were washed twice with 75 mM Tris–65 mM NaCl (pH 7.4) and then incubated on ice with 1 mM diisopropyl fluorophosphate (DIFP; Sigma Chemical Co., St. Louis, Mo.). Amebae were then solubilized by boiling in electrophoresis treatment buffer containing 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (1 mM), *p*-(chloromercuri)phenylsulfonic acid (1 mM), leupeptin (2 μM), *N*-ethylmaleimide (5 mM), and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 1 mM] (all from Sigma) (10). After being boiled for 90 s, the mixture was immediately placed on ice until it was used.

Affinity chromatography with the H8-5 monoclonal antibody. The mouse H8-5 monoclonal antiamebic antibody was purified from cell culture supernatants on a protein A-agarose column (Sigma) and was bound to Affi-Gel 10 as described elsewhere (Petri et al., in press). Amebae were solubilized in 0.1% Triton X-100–50 mM Tris–10 mM EDTA–150 mM NaCl–50 mM KI (pH 8.0), with insoluble debris removed by centrifugation at 10,000 × *g* for 10 min, and then applied to the affinity column. After the column was washed in the buffer described above until no amebic proteins were detected by A_{280} in the flowthrough, the column was eluted with 50 mM glycine (pH 2.5).

SDS-polyacrylamide gel electrophoresis (PAGE). Amebic proteins were electrophoresed on 9, 10, or 12% separating gels and 4% stacking gels made from a monomer stock solution containing 30% acrylamide and 0.8% *N*,*N*-methylenebisacrylamide (Bio-Rad Laboratories, Richmond, Calif.) at 10^4 DIFP-treated trophozoites per lane. Samples and prestained high-molecular-weight standards (Diversified Bioproducts, Newton Center, Mass.) were electrophoresed at 10 to 20 mA for 5 to 8 h.

Western blot (immunoblot) studies with human immune or control sera. Proteins were transferred to 0.1-μm-pore-size nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) by using the Western blot technique of Towbin et al. (18). Additional protein-binding sites on the nitrocellulose were blocked by overnight incubation at 4°C with blot washing buffer (BWB) (50 mM Tris, 200 mM NaCl, 0.1% Tween 20 [pH 7.35]) containing 5% nonfat dry milk (Carnation). Before being used, the nitrocellulose was cut into strips corresponding to the protein lanes and washed in BWB.

Human immune or control serum diluted 1:200 in BWB with 5% nonfat dry milk was used as the primary antibody and incubated with nitrocellulose strips containing amebic protein equivalent to 10^4 trophozoites for 2 h at 37°C. After being extensively washed in BWB, a secondary antibody of peroxidase-conjugated goat anti-human immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, Pa.) was added at a 1:1,000 dilution in BWB with 5% nonfat dry milk for 1 h at 37°C. After being extensively washed, the immunoblots were reacted with 3,3′-diaminobenzidine in 0.05 M Tris (pH 7.2) in the presence of hydrogen peroxide (Sigma).

Immunoprecipitation with H8-5 monoclonal antibody and PHIS. Amebae (10^7/ml) were metabolically labeled by incubation with [^{35}S]methionine (200 μCi/ml; ICN Pharmaceuticals Inc., Irvine, Calif.) for 2 h at 37°C. Amebae (10^7/ml) were solubilized in immunoprecipitation buffer (0.5% Triton X-100, 50 mM Tris, 10 mM EDTA, 150 mM NaCl, 50 mM KI, 20 mM methionine [pH 8.0]) with 1.0% Triton X-100 plus 5 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, and DIFP (1:1,000) and microfuged for 10 min. A total of 1 ml of solubilized amebae was combined with 50 μl of purified H8-5 antibody (50 μg) or 100 μl of PHIS and incubated for 1 h at 4°C. Protein A-agarose beads (Sigma) were then added, and the mixture was incubated for 1 h at 4°C on a rotating platform. Protein A beads were washed extensively, boiled in 100 μl of electrophoresis sample buffer, and loaded onto a 10% SDS gel for electrophoresis. Autoradiography was done by impregnating the gel with Fluoro-Hance (Research Products International, Mount Prospect, Ill.) and exposing it to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) for 3 days at −70°C.

Effect of immune sera on adherence by *E. histolytica*. Adherence of amebae to CHO cells was studied by monitoring rosette formation, as previously described (12, 14). Amebae (10^5/ml) were incubated at 4°C for 30 min with different dilutions of sera and then were washed twice with iced, freshly prepared serum-free Medium 199 (GIBCO) supplemented with 5.7 mM cysteine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and 0.5% bovine serum albumin (Sigma) adjusted to pH 6.8. Amebae (10^4) and CHO cells (2 × 10^5) were suspended in 1.0 ml of the supplemented Medium 199, centrifuged at 150 × *g* for 5 min, and then incubated at 4°C for 2 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 (referred to as an ameba-CHO cell rosette) was determined (12).

Statistics. In all figures, the migration of molecular weight standards is indicated in the left margin. The molecular weights of the amebic proteins were determined by a logarithmic plot with molecular weight standards. All comparisons of significance were by paired or unpaired Student's *t* test as appropriate; unless stated otherwise, data are presented as the mean (± standard error of the mean).

RESULTS

Immunoblots of whole amebic protein and immunoaffinity-purified Gal/GalNAc-binding lectin. Immunoblots with human sera from eight patients treated for amebic liver abscess are shown in Fig. 1. A 170-kDa protein present on SDS-PAGE of DIFP-treated amebae was recognized by all eight immune sera and appeared as the most prominent antigen. None of the eight control sera studied recognized amebic proteins. Immunoblots of sera from five patients studied at 1-year intervals posttreatment for amebic liver abscess demonstrated that recognition of the 170-kDa protein persisted for up to 30 months posttreatment (data not shown). A positive immunoblot of the purified lectin with PHIS demonstrated that the 170-kDa protein recognized by human sera was in fact the Gal/GalNAc-binding lectin (Fig. 2).

Immunoprecipitation with monoclonal antibody H8-5 and human immune sera. To further demonstrate the antigenicity of the Gal/GalNAc-binding lectin, [^{35}S]methionine-metabolically-labeled amebae were solubilized with Triton X-100 and subjected to immunoprecipitation with the antilectin monoclonal antibody H8-5 and PHIS. SDS-PAGE with autoradiography of the immunoprecipitate demonstrated that PHIS and H8-5 immunoprecipitate metabolically labeled amebic proteins of identical molecular weights (170 kDa) (Fig. 3). It is also apparent that among the many metabolically labeled proteins recognized by PHIS, the 170-kDa lectin is the most prominent (Fig. 3).

Effect of human immune sera on amebic adherence. To correlate antibody recognition with inhibition of function of

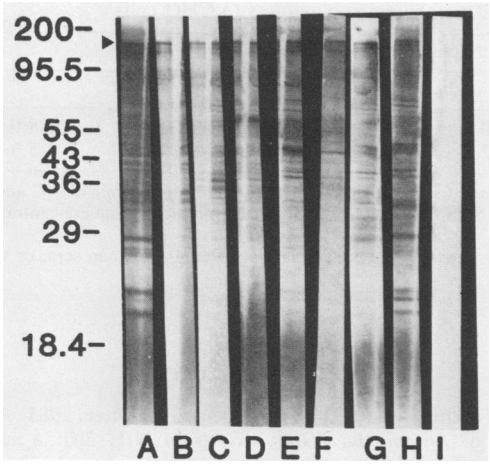

FIG. 1. Immunoblots of amebic protein with eight human immune sera. SDS-PAGE was run with a 10% acrylamide separating gel, and the protein was transferred to nitrocellulose. Lanes A to H, Individual immune sera from patients treated for amebic liver abscess; lane I, representative human control serum. A 170-kDa protein (arrowhead) is prominently recognized by all of the immune sera.

the Gal/GalNAc-binding lectin, we studied the effects of PHIS on parasite adherence to target CHO cells in vitro. Adherence of axenic *E. histolytica* trophozoites (HM1-IMSS) to CHO cells is exclusively mediated by the amebic Gal/GalNAc-binding lectin (12, 14). Amebae (10^5/ml) were exposed to PHIS or control serum for 30 min at 4°C and washed, and adherence to CHO cells was studied by monitoring rosette formation (12) in fresh test medium without added serum. PHIS inhibited parasite adherence at ≥1.0% ($P < 0.01$; Table 1). Control human serum was studied at up to 10% by volume and had no effect on parasite adherence. A dose response for the inhibition of amebic adherence with PHIS was observed (Table 1); exposure to 10% immune serum almost completely inhibited parasite adherence (to 5% of control; Table 1).

DISCUSSION

This study demonstrates that immune sera from patients treated for amebic liver abscess recognized the Gal/GalNAc-adherence lectin of the parasite and inhibited its function in vitro. Since parasite adherence capability is apparently a prerequisite for pathogenesis of invasive amebiasis, the blocking antibody present in immune sera may have an important role in host resistance.

In a previous report, we determined which amebic proteins in a soluble whole-cell preparation were recognized on Western blots with immune sera from liver abscess patients and found that 37-, 59-, and 90-kDa amebic proteins were the most frequently recognized antigens (M. P. Joyce and J. I. Ravdin, Am. J. Trop. Med. Hyg., in press).

In this study, we used preincubation of amebae with the protease inhibitor DIFP (10) to inhibit degradation of the lectin and SDS-PAGE conditions optimal for a 170-kDa amebic protein. The eight immune sera studied all recognized a 170-kDa amebic protein; such recognition persisted for up to 30 months posttherapy in five individuals. Immunoblotting of monoclonal antibody affinity-purified amebic lectin with immune sera confirmed that the human immune sera recognized the Gal/GalNAc-binding lectin.

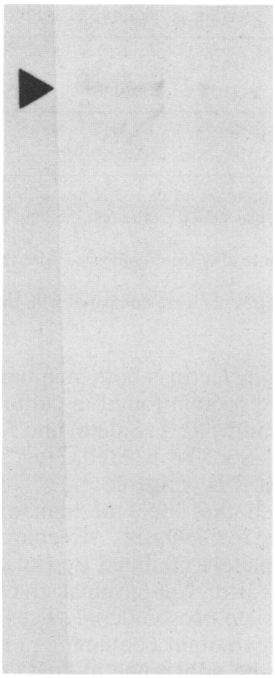

FIG. 2. Immunoblot of the purified Gal/GalNAc adherence lectin with PHIS. Two dilutions of the 170-kDa amebic adherence lectin were subjected to SDS-PAGE, transferred to nitrocellulose, and exposed to a 1:1,000 dilution of pooled sera from patients recovered from amebic liver abscess. The position of the 170-kDa adherence lectin is marked with an arrowhead.

Immunoprecipitation of [^{35}S]methionine-labeled amebic proteins with H8-5 and PHIS demonstrated that the 170-kDa lectin was the major metabolically labeled *E. histolytica* protein recognized by human immune sera. Since the

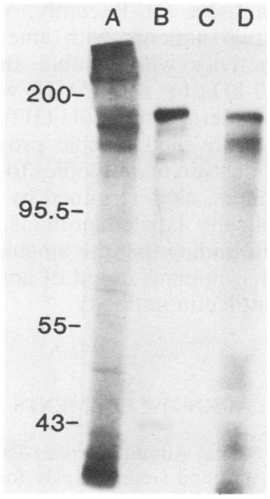

FIG. 3. Immunoprecipitation of [^{35}S]methionine-metabolically-labeled amebic protein with H8-5 and PHIS. Autoradiograph of SDS-PAGE (10% acrylamide separating gel) of [^{35}S]methionine-labeled amebic proteins (A) immunoprecipitated with monoclonal antibody H8-5 (B), normal human serum (C), or PHIS (D). A 170-kDa metabolically labeled amebic protein was immunoprecipitated by lectin-specific H8-5 and PHIS and is the most intensely labeled antigen recognized by PHIS.

TABLE 1. Effect of human immune sera on adherence of *E. histolytica* trophozoites to CHO cells

Pooled human serum	% Rosette formation[a] with the following serum concn (%):				
	0.5	1.0	2	4	10
Control	ND	98.4 ± 1.2	100.9 ± 1.4	102.3 ± 1.9	88.9 ± 5.7
Immune	101.6 ± 9.5	56.4 ± 6.4[b,c]	42.5 ± 3.1[b]	33.5 ± 6.6[b]	5.3 ± 0.6[b,c]

[a] Expressed as a percentage of paired result without prior exposure. Amebae were exposed to the indicated dilution of sera for 30 min at 4°C and washed extensively; no serum was added to the test medium during the 2-h adherence assay at 4°C. Six to eight samples were used for each serum concentration. ND, Not done.

[b] P was <0.01 when results were compared with those without prior exposure to sera, with equivalent concentrations of control human sera, or with 0.5% immune serum.

[c] P was <0.01 when results were compared with those with 2 or 4% immune serum.

Gal/GalNAc-binding lectin is both membrane associated and the major released protein found in culture medium (W. A. Petri, Jr., R. D. Smith, R. A. Salata, and J. I. Ravdin, Abstr. Annu. Meet. Am. Soc. Trop. Med. Hyg. 1986, no. 41), it is not surprising that it is antigenic.

We also studied the effect of human immune sera on parasite adherence to CHO cells. Amebic adherence to CHO cells can be completely inhibited by Gal or GalNAc monomers (1.0 g %) (12) or Gal-terminal glycopeptides such as asialofetuin or asialo-orosomucoid (Petri et al., in press). Tissue culture supernatant containing monoclonal antibody F14 inhibits parasite adherence to CHO cells by 86% (15). Exposure of amebae at 4°C to human immune sera at ≥1.0% inhibited subsequent adherence to CHO cells, and 95% inhibition was observed after incubation with 10% immune sera. Control human sera at these concentrations had no effect on adherence. These observations by themselves may not be entirely specific for antilectin antibody, since antibodies to other amebic surface proteins (1; Joyce and Ravdin, in press) could contribute to inhibition of lectin function by steric hindrance.

Aust-Kettis and co-workers (1) studied the antigens of *E. histolytica* NIH 200, a less-virulent strain (9) containing less biologically active Gal/GalNAc-binding lectin than strain HM1-IMSS (14). These researchers found that an amebic protein of approximately 150 kDa was recognized by a pool of eight immune antisera (1). Recently, Mathews et al. (8), using sera from two patients with amebic liver abscess, demonstrated reactivity with soluble amebic proteins of approximately 170 kDa by SDS-PAGE with gradient polyacrylamide gels. Calderon and Avila (3) found that immunization of rabbits with shed amebic protein (strain HM1-IMSS) elicited production of antibodies to a 170-kDa amebic protein, among others, as determined by immunoprecipitation with metabolically labeled proteins. Therefore, prior studies support our finding that the amebic adherence lectin is antigenic and that humans cured of amebic liver abscess possess specific antilectin antibody.

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