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Production of Mouse Monoclonal Antibodies Which Inhibit In Vitro Adherence of *Entamoeba histolytica* Trophozoites

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Adherence by axenic *Entamoeba histolytica* trophozoites to mammalian cells is mediated by an *N*-acetylgalactosamine (GalNAc)-inhibitable adhesin on the surface of the parasite. We isolated 35 hybridoma cell lines producing antibodies to *E. histolytica* as indicated by ELISA with sonicated amebic protein or by immunofluorescence assay with fixed whole trophozoites. Tissue culture supernatants were further screened for subcloning by the ability to bind to Chinese hamster ovary (CHO) cells which were first exposed to a partially purified soluble preparation of the amebic GalNAc-inhibitable lectin. Eight tissue culture supernatants were positive in this assay. Antibodies from four subcloned cell lines (D3-14, H8-5, I12-2, and I1-21) inhibited amebic adherence to CHO cells ($P < 0.01$). Of the original 35 tissue culture supernatants, 3 also inhibited amebic adherence ($P < 0.01$; F1, F14, and J10); monoclonal antibodies in these supernatants did not bind to lectin-exposed CHO cells. Three purified monoclonal antibodies (H8-5, I12-2, and I1-21) inhibited amebic adherence at ≥2 μg/10^4 amebae ($P < 0.05$). None of these inhibitory monoclonal antibodies immunoprecipitated with a soluble amebic protein preparation following sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions. Monoclonal antibodies which inhibit in vitro adherence by *E. histolytica* will be useful in purification of the GalNAc-inhibitable lectin.

Amebiasis is an important worldwide disease caused by infection by the cytolytic enteric protozoan *Entamoeba histolytica*. In vivo or in vitro intestinal models of amebiasis indicate that amebae adhere to host inflammatory cells and the intestinal mucosa before invasion (7, 11, 15). A surface adhesin of *E. histolytica* which is inhibitable by *N*-acetylgalactosamine (GalNAc) or galactose-terminal oligosaccharides mediates adherence of the parasite to Chinese hamster ovary (CHO) cells (17, 19), human erythrocytes (17), human neutrophils (19), opsonized bacteria or bacteria whose lipopolysaccharide contains galactose (2, 3), and fixed rat or human colonic mucosa (18). Soluble protein preparations of *E. histolytica* contain soluble lectin inhibitable by GalNAc or galactose, as assayed by agglutination of CHO cells or erythrocytes (19). This soluble lectin is most likely homologous with the surface adhesin. Adherence of axenic *E. histolytica* trophozoites to cells or tissue is a prerequisite for amebic cytolytic activity (14, 16, 17). GalNAc inhibits amebic lysis of target CHO cells and human neutrophils (17, 19). Immunization of rabbits with a partially purified preparation of this lectin elicited immune sera which partially inhibited adherence of amebic trophozoites to rat colonic mucosa in an in vitro organ culture model (18).

The purpose of this study was to produce monoclonal antibodies to *E. histolytica* proteins and to identify antibodies which inhibit amebic adherence to CHO cells. Inhibitory monoclonal antibodies may be recognizing epitopes on the amebic GalNAc-inhibitable lectin (adhesin) molecule.

MATERIALS AND METHODS

Cultivation of *E. histolytica* trophozoites. Axenic *E. histolytica* HM1-IMSS trophozoites were provided by D. L. Diamond (National Institutes of Health, Bethesda, Md.) and have been maintained in our laboratory for about 6 years. Amebae were grown in B-S-33 (Biosate [BBL Microbiology Systems, Cockeysville, Md.], iron, and serum) medium as developed by Diamond et al. (9), containing penicillin (100 U/ml) and streptomycin sulfate (GIBCO Laboratories, Grand Island, N.Y.) (100 μg/ml). Amebic cultures were maintained and harvested as described previously (15, 16).

Generation of mouse monoclonal antibodies to *E. histolytica*. BALB/c mice were inoculated intramuscularly with 5×10^5 sonicated *E. histolytica* trophozoites in complete Freund adjuvant. After two intraperitoneal booster injections of sonicate (10^5 amebae) at 3 and 5 weeks, mice with the highest antibody titers (see below) to *E. histolytica* were sacrificed 4 days after an intravenous injection of amebic sonicate (10^4 trophozoites). The spleen cells were fused with P3 × 63-Ag8 mouse myeloma cells at a ratio of 5:1 to 7:1 using 1,500 M_w polyethylene glycol (Baxter, Grand Prairie, Tex.) as the fusing agent (12). Cells were then suspended in Dulbecco medium (GIBCO) containing 20% fetal bovine serum, 0.1% gentamicin sulfate, hypoxanthine (10^{-4} M), aminopterin (4×10^{-7}), and thymidine (1.6×10^{-5} M) (HAT medium) placed in 96-well microtiter plates (Costar, Cambridge, Mass.) and incubated at 37°C in 10% CO_2. Samples of tissue culture fluid obtained at 3 to 6 weeks from wells with proliferating clusters of cells were screened for antibody to *E. histolytica* by solid-phase immunoassay with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG) (Zymed Laboratories, South San Francisco, Calif.) as the indicator system enzyme-linked immunosorbent assay [ELISA]) and by indirect immunofluorescence assay with whole acetone-fixed amebic trophozoites with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin, H chain specific (Cooper Biomedical, Inc., West Chester, Pa.), as the label. For subcloning by limited dilution, approximately 100 hybridoma cells from a single well were distributed to 96 wells, each containing 10^5 mouse thymic cells as a feeder

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layer. The supernatants from wells with single clones were tested for antibody by ELISA, immunofluorescence assay, and a modified CHO cell assay. The immunoglobulin subclass (IgG1, IgG2a, IgG2b, IgG3, and IgM) present in each tissue culture supernatant was determined by an enzyme-linked immunoassay (mouse monoclonal subisotyping kit; HyClone Laboratories, Logan, Utah).

Screening of antibodies by binding to lectin-exposed CHO cells. A partially purified preparation of the amebic GalNAc-inhibitable lectin was prepared from HM1 amebae by sonication, ultracentrifugation, and gel filtration chromatography (Sephacyl S-300; Pharmacia Fine Chemicals, Piscataway, N.J.) in a 19 mM phosphate–67 mM NaCl buffer (pH 7.2) as described previously (19). CHO cells, grown in F-12 medium (GIBCO) as described previously (19), were washed twice in Dulbecco phosphate-buffered saline (GIBCO) and fixed with 1% glutaraldehyde in phosphate-buffered saline for 20 min. Fixed CHO cells (2×10^5/ml) were washed twice in the 19 mM phosphate buffer and incubated at 4°C for 60 min in the partially purified lectin preparation (100 μg/ml). Lectin-exposed and control CHO cells were again washed twice in the 19 mM phosphate buffer and then incubated in a 1:5 dilution of tissue culture supernatant, containing mouse monoclonal antibodies, for 60 min at 4°C. The CHO cells were washed twice, and the binding of mouse monoclonal antibody to lectin-exposed or control CHO cells was determined by fluorescence microscopy with fluorescein-conjugated rabbit anti-mouse immunoglobulin (1:40 dilution).

Effect of monoclonal antibodies on amebic adherence to CHO cells. Adherence of amebae to CHO cells was studied by rosette formation as previously described (17, 19). Amebae (10^5/ml) were incubated at 4°C for 60 min in 100% tissue culture supernatant or partially purified mouse monoclonal antibodies at various concentrations. Amebae 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 Chemical Co., St. Louis, Mo.) 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 $150 \times g$ 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 (17).

Purification of monoclonal antibodies. Monoclonal antibodies of the IgG subtypes were isolated by affinity chromatography with staphylococcal protein A covalently attached to Sepharose CL-4B (Sigma) (10). IgM antibodies were purified by precipitation by dialysis against distilled water at 4°C followed by gel filtration on Sephacryl S-300 (Pharmacia).

Statistics. All comparisons of significance were made by paired or unpaired Student's *t* test as appropriate; unless stated otherwise, data is presented as the mean ± standard error of the mean.

RESULTS

Five fusions resulted in 288 wells containing proliferating hybridoma cells, of which 35 were positive by either ELISA with amebic proteins or by immunofluorescence assay to fixed whole trophozoites (Table 1). A total of 24 (68.6%) of the 35 were positive by ELISA, 19 (54.3%) of the 35 were positive by immunofluorescence assay, and 8 (22.9%) of the 35 were positive by both screening methods. We determined type or subtypes of immunoglobulin present in culture supernatants before subcloning (Table 1). Unfortunately, at up to 1:100 dilution, tissue culture supernatants that did not contain antibody gave a false positive result in the CHO cell agglutination assay. Therefore, supernatants could not be screened directly in that assay for inhibition of the soluble amebic GalNAc-inhibitable lectin. To determine which monoclonal antibodies may be binding to the lectin molecule, fixed CHO cells were exposed to a partially purified preparation of the amebic lectin (19), and nonadherent amebic proteins were washed off. Binding of monoclonal antibodies to the lectin-exposed CHO cells was determined by immunofluorescence assay with fluorescein-conjugated rabbit anti-mouse immunoglobulin. Fixed CHO cells which were not exposed to the lectin preparation served as a control.

Of the 35 monoclonal antibodies, 8 bound to lectin-exposed CHO cells and not to control CHO cells (Table 1). Parent clones were chosen for subcloning based on their binding to lectin-exposed CHO cells. Immunoglobulins from 9 subclones were subsequently identified to bind to lectin-exposed CHO cells (Table 2).

All original parent clone supernatants and those from the subclones positive in the CHO cell-binding assay were

TABLE 1. Parent hybridoma clones which produced antibody to *E. histolytica*

Fusion date (1983)	Designation	Immunoglobulin subclass	ELISA	Immunofluorescence[a]	Binding to exposed CHO cells
6/8	I-1	ND[b]	+	+	ND
6/21	D-3	ND	+	−	ND
	F-1	ND	+	−	ND
10/18	A-1	IgG1	+	−	−
11/3	E-1	ND	+	−	+
	H-6	IgG1	+	−	+
	I-4	IgG1	−	+	−
11/17	B-4	IgG1	+	+	−
	B-5	ND	+	−	−
	C-6	IgM	+	−	ND
	D-3	IgG1	−	+	−
	D-10	IgG1	+	−	−
	D-11	IgM	−	+	−
	H-8	IgG2b	−	+	+
	H-12	IgG1,2a,2b	+	−	−
	I-1	IgG2b + IgG2b	+	+	−
	I-3	ND	+	+	+
	I-5	IgG1 + IgG2b	+	+	−
	I-8	ND	+	+	−
	I-12	ND	+	+	+
	J-4	IgG1	+	−	−
	J-8	IgM + IgG1	+	+	−
	J-10	IgG1	−	+	−
	J-22	IgG1 + IgG2b	−	+	−
	E-7	IgM	+	−	+
	E-13	IgG1	−	+	−
	E-14	IgG1	−	+	−
	F-1	IgM + IgG2a	+	−	−
	F-4	IgG1	−	+	−
	F-8	IgG1	+	−	−
	F-9	IgG1	−	+	+
	F-12	IgG1	−	+	−
	F-14	IgM	+	−	−
	G-8	IgG1	+	−	−
	G-10	IgG1,2a,2b,3	+	−	+

[a] Performed with acetone-fixed trophozoites.
[b] ND, Not done.

TABLE 2. Subcloned monoclonal antibodies which bound to lectin-exposed CHO cells

Designation	Immunoglobulin subclass	ELISA	Immunofluorescence[a]
D3-14	IgG1	+	+
E1-1	IgM	+	−
F9-4	IgG1	−	+
G10-12	IgG2a	+	−
H6-4	IgG1	+	−
H8-5	IgG2b	−	+
I1-21	IgM	+	+
I3-4	IgM + IgG2a	+	+
I12-2	IgM	+	−

[a] Performed with acetone-fixed trophozoites.

studied for their effect on adherence of viable *E. histolytica* trophozoites to CHO cells. Amebae were incubated in the undiluted tissue culture supernatant for 60 min at 4°C and were washed, and then adherence to CHO cells was studied at 4°C in supplemented medium 199 without the presence of the tissue culture supernatant. Exposure of amebae to sham tissue culture supernatant or tissue culture supernatant containing nonrelated monoclonal antibodies (to murine cytomegalovirus) had no effect on adherence ($n = 8$ for each). A total of 3 of the 35 parent hybridoma supernatants and 4 of the 9 selected subclone supernatants inhibited adherence of *E. histolytica* trophozoites to CHO cells (Table 3; all at $P < 0.01$ compared with simultaneous paired control studies, $n \geq 6$ for each of the 32 parent and 5 subclone supernatants that were not inhibitory).

The three parent clone supernatants (F1, F14, and J10) which inhibited adherence did not bind to lectin-exposed CHO cells. Although F1 and F14 contained IgM, F1 also contained IgG2a and J10 had solely IgG1; numerous other IgM antibodies had no effect on amebic adherence (Table 1) indicating specificity and not inhibition based on steric interference or other general physical properties of IgM antibodies. F1 and F14 were negative by immunofluorescence assay to fixed amebic trophozoites, however, both were positive when studied with live trophozoites at 4°C (Table 3).

Selection of subclones based on their binding to lectin-exposed CHO cells resulted in the isolation of four subclones (D3-14, H8-5, I12-2, and I1-21) which inhibited amebic adherence. All subclones bound at 4°C to the surface of viable *E. histolytica* trophozoites (Table 3). F1 has been

TABLE 3. Monoclonal antibody-containing supernatants which inhibited adherence by *E. histolytica* trophozoites to CHO cells

Hybridoma designation	Immunoglobulin subclass	ELISA	Immunofluorescence		% Inhibition of amebic adherence to CHO cells[a] (n)
			Acetone fixed amebae	Viable amebae at 4°C	
D3-14	IgG1	+	−	+	67.0 ± 2.1[b] (16)
F1	IgM, IgG2a	+	−	+	21.3 ± 6.0 (6)
F14	IgM	+	−	+	86.3 ± 1.7[b] (14)
H8-5	IgG2b	−	+	+	34.3 ± 3.3 (14)
I1-21	IgM	+	+	+	29.4 ± 3.3 (14)
I12-2	IgM	+	−	+	38.3 ± 2.7 (14)
J10	IgG1	−	+	+	26.1 ± 2.6 (14)

[a] Determined by rosette formation after exposure of amebae to supernatant at 4°C, compared with paired control studies, $P \leq 0.01$ for each antibody.
[b] $P < 0.01$ compared with all other inhibitory supernatants.

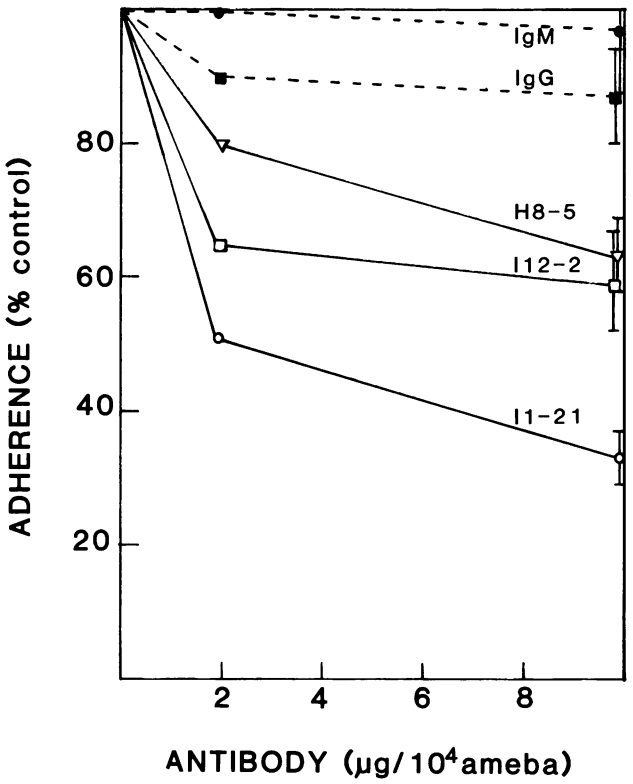

FIG. 1. Inhibition of amebic adherence to CHO cells by purified monoclonal antibodies. Amebae were exposed to purified H8-5, I12-2, and I-21 for 60 min at 4°C in medium 199. Data is presented as percentage of adherence observed for control amebae not exposed to antibody. IgM and IgG were control unrelated monoclonal antibodies obtained commercially (Sigma). Exposure of amebae to H8-5, I12-2, or I1-21 at 2 or 10 μg/10^4 amebae followed by washing resulted in inhibition of adherence ($P < 0.05$ for each); control IgG or IgM had no significant effect.

subcloned (F1-1 to F1-10, all IgG2a), but none of the tissue culture supernatants from these subclones inhibited amebic adherence to CHO cells, suggesting that the F1 IgM is responsible for the inhibition observed with the parent hybridoma supernatant and that further subcloning of F1 is indicated. Under the conditions studied, tissue culture supernatants from the parent cultures (D3, H8, I1, and I12) of the four inhibitory subclones did not inhibit amebic adherence.

To date, we have been successful in purifying three of the

TABLE 4. Inhibition of amebic adherence to CHO cells by purified monoclonal antibodies

Antibody (2 μg/10^4 amebae)	% Adherence[a]
Control antibody (IgM)	100 ± 6.0
H8-5	80 ± 9.0
I12-2	65 ± 6.0
I1-21	51 ± 11.0
I12-2 + I1-21	69 ± 4.0
I12-2 + H8-5	38 ± 5.0[b]
I1-21 + H8-5	69 ± 10.0

[a] Data expressed as a percentage of the rosette formation observed without prior exposure of the amebae to antibody.
[b] $P < 0.01$ compared with H8-5 or I12-2.

inhibitory subcloned monoclonal antibodies (H8-5, I12-2, and I1-21) from large volumes of tissue culture supernatant. Unfortunately, the cell line producing monoclonal antibody with the most inhibitory cell supernatant, F-14, is no longer viable. Exposure of amebae at 4°C to purified H8-5 (IgG2b), I12-2 (IgM), and I1-21 (IgM), followed by washing, resulted in decreased adherence to CHO cells at as low as 2 μg of antibody per 10^4 amebae (Fig. 1, $P < 0.01$). Control nonrelated monoclonal IgG or IgM (Sigma) had no effect on adherence by *E. histolytica* trophozoites. At 2 μg/10^4 amebae, H8-5 and I12-2 in combination resulted in an additive inhibitory effect on adherence (Table 4). The combination of I12-2 and I1-21 or I1-21 and H8-5 was no more inhibitory than each monoclonal antibody alone (Table 4).

Immunoblotting was attempted with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of a soluble preparation of amebic sonicate (19) transferred to nitrocellulose and probed with the tissue culture supernatants which inhibited adherence (F1, F14, J10, H8-5, I12-2, I1-21, and D3-14) (20). None of the monoclonal antibodies present in these tissue culture supernatants definitively recognized *E. histolytica* proteins under the denaturing conditions of the SDS-polyacrylamide gel electrophoresis. This was true even if the proteins were solubilized in SDS without boiling and without the reducing agent (BME) present. Dot blots of native amebic protein on nitrocellulose probed with tissue culture supernatants were reactive for all supernatants of the inhibitory monoclonal antibodies. Use of three purified monoclonal antibodies (H8-5, I12-2, and I1-21) gave identical results.

DISCUSSION

This is to our knowledge the first report of the isolation of monoclonal antibodies to *E. histolytica* which are capable of inhibiting in vitro adherence by the parasite.

ELISA and immunofluorescence with acetone-fixed trophozoites were used as the initial screening assay to choose clones which produced antibody to amebic proteins. Both methods were complementary in that less than a quarter of the tissue culture supernatants containing antibody were positive by both ELISA and immunofluorescence assay. The amebic GalNAc-inhibitable lectin appears to be a soluble lectin (1) as it is not an integral membrane protein and can be washed off amebae with high-salt buffers (19). Binding of a monoclonal antibody to fixed trophozoites assayed by immunofluorescence was not predictive of the antibody binding to lectin-exposed CHO cells or having the capacity to inhibit amebic adherence. Obviously, for a monoclonal antibody to inhibit adherence, it should be binding to the surface of the parasite. In retrospect, it would have been better to use live trophozoites at 4°C rather than fixed cells. Monoclonal antibodies D3-14, F1, F14, and I12-2 inhibited adherence but did not bind to acetone-fixed amebae. However, these antibodies did bind to live trophozoites at 4°C. The amebic adherence protein is glutaraldehyde sensitive (18, 19) and may be lost or altered following fixation with acetone.

As the surface GalNAc-inhibitable adhesin and the soluble lectin are likely homologous molecules (19), one strategy for selecting inhibitory antibodies was to identify monoclonal antibodies which bound to the soluble lectin molecule. We had hoped to screen monoclonal antibodies directly in the CHO cell agglutination assay (19) for inhibition of the soluble amebic lectin. Unfortunately, even low concentrations of non-antibody-containing tissue culture supernatants gave false positive results (agglutinated CHO cells). Serum proteins and large macromolecules have a similar effect (19). In response to this problem, we devised an assay making use of the CHO cell as a presumed affinity carrier for the amebic lectin molecule. However, additional amebic proteins could be absorbed to the CHO cell surface and result in a lack of specificity for this method. Four of nine subcloned antibodies positive in this assay inhibited amebic adherence, suggesting some utility for this screening method. However, this assay did not identify three hybridoma supernatants containing monoclonal antibodies (F1, F14, and J10) which inhibited adherence by viable trophozoites. One speculation is that with the amebic lectin bound to its receptor on the CHO cell, its carbohydrate binding domain was not optimally available for recognition by IgM monoclonal antibodies, as two of the three inhibitory parent hybridomas produced IgM.

Inhibition of amebic adherence mediated by the GalNAc-inhibitable adhesin molecule blocks amebic cytolytic activity (17), and in different strains of axenic amebae the lectin activity per milligram of protein or per cell correlated with in vitro virulence (19). Intestinal mucus may bind amebae by mechanisms independent of the GalNAc-inhibitable lectin (13, 18). Inhibition of the surface adhesin by GalNAc makes the ameba more vulnerable to human effector cells such as neutrophils. Lysis of human neutrophils, due to a mechanism dependent on the GalNAc-inhibitable adhesin, potentiates the parasite's destruction of host liver cells in vivo and in vitro (21; R. A. Salata, and J. I. Ravdin, J. Infect. Dis., in press).

Seven monoclonal antibodies (F1, F14, J10, H8-5, I12-2, I1-21, and D3-14) inhibited adherence of axenic *E. histolytica* trophozoites to CHO cells, with cell supernatant from F-14 providing almost complete (86%) inhibition. These antibodies are of the IgM (F1, F14, I1-21, I12-2), IgG1 (D3-14, J10), or IgG2b (H8-5) class. Although F1 also contained IgG2a, 10 IgG2a subclones from F1 were not inhibitory, indicating that the F1 IgM is relevant to inhibition. The specificity of our observations is supported by the different classes of immunoglobulin which were inhibitory, and by the multiple other monoclonal antibodies of the same classes which were positive by ELISA or immunofluorescence assay to *E. histolytica* but which had no effect on amebic adherence. The additive inhibitory effects on adherence of H8-5 (on IgG2b) and I12-2 (on IgM) suggest that these antibodies are binding to different sites on or near the surface adhesin. I1-21 (an IgM) may be recognizing identical epitopes as either I12-2 or H8-5 or has a near enough binding site which results in steric hindrance of antibody binding. Previous studies using rabbit immune sera, elicited by immunization with a partially purified but still complex preparation of the amebic lectin (19), at 5.0% provided only 22% inhibition of amebic adherence (18). The high magnitude of inhibition observed with microgram concentrations of the monoclonal antibodies described in this report supports their increased specificity and future utility.

Unfortunately, the antibodies in cell supernatants which inhibited adherence did not bind in Western blots to amebic proteins from an ultracentrifuged sonicate fraction separated by SDS-polyacrylamide gel electrophoresis (5). This is a frequent observation regarding denatured proteins and monoclonal antibodies (4, 22); a relevant example was a study of monoclonal antibodies to the protozoan *Trichomonas vaginalis* (8). In this study, native amebic protein was used to produce monoclonal antibodies; these antibodies may be binding to epitopes which are no longer recognizable when the protein is denatured in SDS (4). Use of a denatured

protein as an immunogen (22) might be advantageous for Western blot studies. However, carbohydrate-binding activity of the amebic lectin (adhesin) molecule is lost following denaturation in SDS (unpublished data). Therefore, use of denatured amebic protein to immunize mice may not have produced antibodies which inhibited the lectin-mediated adherence by viable trophozoites.

Monoclonal antibodies to other protozoa have been isolated which can inhibit virulence of the organism, such as with *Babesia bovis* (23). The antibodies described in this report may be binding directly to the adhesin molecule or to nearby amebic antigens causing steric interference with adherence by the parasite. Further purification of the amebic lectin and immunoprecipitation (4) will be necessary to determine whether all the inhibitory monoclonal antibodies described in this report are binding to an identical amebic protein. Immunoaffinity chromatography with the purified inhibitory monoclonal antibodies has the potential to isolate the adherence lectin of *E. histolytica*. Further characterization of the adhesin on whole amebae and purification of the soluble lectin with its use as an immunogen to produce protective antibody may lead to a means of prevention of in vivo disease. The monoclonal antibodies described in this report may have other applications in clinical diagnosis or for description of additional amebic molecules that have a role in pathogenesis of amebiasis.

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