Role of Adherence in Cytopathogenic Mechanisms of *Entamoeba Histolytica*

STUDY WITH MAMMALIAN TISSUE CULTURE CELLS AND HUMAN ERYTHROCYTES

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ABSTRACT The enteric pathogen, *Entamoeba histolytica*, appears to cause disease by adhering to and then destroying mucosal barriers. Using an in vitro method of studying the interaction of *E. histolytica* with target cells (Chinese hamster ovary [CHO] and human erythrocytes [RBC]), we examined the mechanism of amebic adherence and its role in lysis of target cells. Killing and phagocytosis of target cells by amebas ceases at 4°C, allowing observation of adherence. Amebas adhere to CHO cells at 4°C, 78.9% formed rosettes (amebas with ≥3 adherent CHO cells each) at 2 h. At 37°C, cytochalasins B and D inhibit adherence of amebas to CHO cells ($P < 0.0005$). Amebas adhere to and kill CHO cells in media with <0.1 µM calcium and magnesium plus 10 mM EDTA, indicating that divalent cations are not required in the medium. Adherence of amebas to human RBC was not ABO blood group specific and showed greater adherence to human than bovine or sheep RBC ($P < 0.005$). Neither Fc nor complement receptors were found on amebas by standard rosette studies. The amebic adherence receptor is not trypsin (0.125%) sensitive nor inhibited by trypan blue (1 mM). N-acetyl-D-galactosamine (GALNAc) inhibited the adherence of amebas to CHO cells and human RBC (0.1 g/100 ml or 4.5 mM GALNAc, $P < 0.005$) by binding to a receptor on the amebic surface. GALNAc abolishes amebic cytolysis of target CHO cells (determined by ^{111}Indium oxine release from CHO cells, $P < 0.001$) but not amebic phagocytosis of CHO cells. By suspend-

ing ameba-CHO cells rosettes in dextran, we found that GALNAc (1%) reversibly inhibits amebic adherence ($P < 0.0005$) and that cytochalasins decrease amebic killing of adherent CHO cells ($P < 0.025$).

These findings indicate that the adherence of *E. histolytica* to target cells requires microfilament function, is via a specific amebic receptor that has affinity for GALNAc, and is required to lyse cells. Inhibition of the adherence of *E. histolytica* may alter the pathogenicity of this organism.

INTRODUCTION

Entamoeba histolytica causes invasive amebiasis by crossing intestinal mucosal barriers. As with other enteric pathogens, one might expect that a critical early event in the pathogenesis of amebiasis would involve the initial adherence of amebas to cell surfaces. Such an initial adherence event would be especially important in an invasive process that appears to involve contact-dependent cell destruction.

In previously reported work (1–6), we and others have demonstrated that *E. histolytica* kills target cells only on direct contact, and that this cytolethal effect occurs at the amebic surface and before phagocytosis. Killing of target cells by amebas requires the function of microfilaments, as evidenced by inhibition with cytochalasins, but not microtubules (3, 4). The purposes of the present studies were to examine the adherence of amebas to target cells (Chinese hamster ovary [CHO][1] cells, and human, bovine, and sheep

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[1] *Abbreviations used in this paper*: BRBC, bovine erythrocytes; CHO, Chinese hamster ovary; Cyto, cytochalasin; Eh, *Entamoeba histolytica*; FCS, fetal calf serum; HRBC, human erythrocytes; ^{111}InOx, ^{111}Indium oxine; PBS, phosphate-buffered saline; PSF, puck saline F; SRBC, sheep erythrocytes; TYI, trypticase-yeast extract-iron.

red blood cells), the role of adherence in the killing of target cells by amebas, and the specificity of the adherence mechanism.

Lectins have been isolated from several unicellular organisms, including the slime mold *Dictyostelium discoideum* (7), and, recently, axenic *E. histolytica* (8). Other workers have noted that human erythrocytes adhere to *E. histolytica* (8, 9).

By selectively inhibiting the prompt, contact-dependent cytolethal effect of *E. histolytica*, we have been able to investigate a prior adherence event. We have found that *E. histolytica* adheres to CHO cells and erythrocytes, and that a specific adherence event is required for amebas to kill target cells (at the amebic surface), but this event is not necessary for phagocytosis by amebas. Microfilament inhibitors, cytochalasins B and D, decrease amebic cytopathogenicity by inhibiting both amebic adherence and killing of target cells following the adherence. Adherence of *E. histolytica* to target cells appears to involve an amebic receptor for specific carbohydrates as it is inhibited by *N*-acetyl-D-galactosamine and is specific for human erythrocytes.

METHODS

Cultivation and harvesting of E. histolytica. Axenic *E. histolytica* (Eh) strain HM 1:IMSS was kindly provided by Dr. L. Diamond of the National Institutes of Health. The amebas were grown in TYI-S-33 medium (trypticase, yeast extract, iron, and serum), as developed by Diamond et al. (10), containing 100 U/ml penicillin (Pfizerpen G, Pfizer Chemicals Div. [Pfizer Inc.,] New York) and 100 μg/ml streptomycin sulfate (Pfizer Inc.). Maintenance and harvesting of Eh cultures was as described previously (3). Amebas were washed twice with freshly prepared, serum-free Puck's saline F (PSF, with pH adjusted to 6.8, and checked after filter sterilization using a 0.2-μm filter, Nalge Co., Nalgene Labware Div., Rochester, N. Y.) before use in adherence assays.

Cultivation and harvesting of CHO cells. CHO cells were grown in F-12 medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum (FCS) (Grand Island Biological Co.), penicillin (100 U/ml), and streptomycin (100 μg/ml) and harvested as described previously (3). Trypsinized (0.25% for 5 min) CHO cells were suspended in test media, washed once, and adjusted to 2×10^5 cells/tube (12 × 75-mm polystyrene tubes, Falcon Labware, Div. of Becton, Dickinson and Co., Oxnard, Calif.). CHO monolayers were prepared as previously described (3) in 24-well, flat bottomed tissue culture plates (Linbro Chemical Co., Hamden, Conn.) and washed twice with TYI (TYI-S-33 without serum) before adding amebas.

Studies of temperature sensitivity of target cell killing by amebas. Amebas (1×10^4) and CHO cells (2×10^5) were suspended in TYI (1% FCS), centrifuged at $150 g$ for 5 min, and then incubated for 2 h at the desired temperature. Pellets were then dispersed with 0.1 ml of 0.4% aqueous trypan blue (Matheson Coleman and Bell, Division of Matheson Co., Inc., Rutherford, N. J.) and the total number of live and dead CHO cells were counted using a hemocytometer chamber as described previously (3).

Details of [111]Indium oxine ([111]InOx) (Diagnostics Isotopes, Inc., Bloomfield, N. J.) labeling of CHO cells have been

described (3). [111]InOx-labeled CHO cells were suspended in TYI (1% FCS) and gamma activity was measured (Beckman Gamma Counter, Beckman Instrument, Inc., Fullerton, Calif.), after which amebas were added and the suspension was centrifuged and incubated as above. At 2 h the supernatants were removed and pellet and supernatant activities were counted separately (then corrected for background and decay from time zero with $e^{-\lambda E}$, where $\lambda = \ln (2/t_{1/2})$, $t_{1/2}$ = 67.2 h). After indium activity was counted, pellets were resuspended in 1.0 ml of fresh TYI (1% FCS) with trypan blue, and hemocytometer counts were made.

Studies of adherence of amebas to CHO cells. Amebas and CHO cells were harvested and washed, as described, in PSF; ~1 h elapsed between trypsinization of CHO cells and their contact with amebas. Amebas (1×10^4) and CHO cells (2×10^5) were suspended in 1 ml of PSF, centrifuged at $150 g$ for 5 min, and then incubated for the desired time, all at 4C. When adherence studies were done at 37°C incubation, centrifugation was done at 23°C. After incubation, 0.8 ml of supernatant was removed, the pellet was broken up by repeated rotation of the tube, and a drop of the cell suspension was placed on a hemocytometer before the cover slip was added. The number of Eh with at least three adherent CHO cells (referred to as an ameba-CHO cell rosette) and the remainder of the amebas were counted. Cytochalasins B and D (Cyto B and D) were diluted in dimethyl sulfoxide (all Sigma Chemical Co., St. Louis, Mo.) to 1,000 μg/ml, and stored at −45°C until used. Upon use, the cytochalasins were further diluted in PSF to final concentrations of 5 or 10 μg/ml. The effect of cytochalasins was evaluated at 4°C (with and without prior Eh incubation in Cyto at 37°C) and at 37°C.

Studies in calcium- and magnesium-free media. PSF was freshly prepared without calcium or magnesium; FCS (Grand Island Biological Co.) was dialyzed (dialysis membrane, Union Carbide Corp., Chicago, Ill.) for 24 h with Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Grand Island Biological Co.) and then added at 1% to PSF. PSF (1% dialyzed FCS) was confirmed to have ≤0.1 μM concentrations of calcium and magnesium by atomic absorption spectrophotometry. EDTA was added to make a 10 mM solution. Studies of adherence at 4°C and target cell killing at 37°C were evaluated after a 2-h incubation. Control preparations were handled similarly except that they contained calcium and magnesium.

Studies of amebic adherence to erythrocytes. Human erythrocytes (HRBC) were obtained fresh from healthy donors of known ABO types. Heparinized blood (2 ml with 100 U of Heparin, Sigma Chemical Co.) was mixed with 2 ml of fresh saline (0.9%), layered above 3 ml of lymphocyte separation medium (LSM, Litton Bionetics, Inc., Kensington, Md.), and then centrifuged for 40 min at 20°C at $400 g$ (11). HRBC were washed twice and diluted in PBS (Grand Island Biological Co.) to 1×10^6/ml. Bovine (BRBC) and sheep (SRBC) blood were obtained from the University of Virginia Vivarium. RBC were separated using lymphocyte separation medium as above for comparison to HRBC, and washed twice in PBS. BRBC and SRBC used in studies of Fc and complement receptors were separated by centrifugation without LSM and then washed five times in PBS. Aliquots of SRBC (2% solution) were incubated with 19S anti-sheep rabbit IgM (1:100 dilution, N. L. Cappel Laboratory, Inc., Cochranville, Pa.) for 30 min, washed three times in PBS, and incubated with fresh mouse serum containing complement (15 min at 37°C). SRBC were then washed again three times before use (12). Aliquots of BRBC (5% solution) were mixed with equal volumes of rabbit antibovine IgG and IgM (1:10 dilution, N. L. Cappel Laboratory Inc.) for 30 min at 37°C, then washed three times in PBS (12).

HRBC, BRBC, and SRBC were diluted to 1×10^6/ml in PBS, suspended with Eh (1×10^4) at 4°C, and centrifuged at 150 g for 5 min at 4°C. Adherence of RBC was evaluated as described for CHO cell adherence. Testing for Eh, Fc, and complement receptors was done by the method of Kay et al. (12); 1% solutions (in PBS) of RBC being tested were centrifuged at 50 g for 5 min at 4°C, and Eh rosette formation was evaluated as above. HRBC (type O, lymphocyte separation medium separated 1×10^6/ml) was used as a control (at 50 g centrifugation) for adherence to amebas.

Studies of trypan blue and trypsin effects. Trypan blue was diluted to 10 mM in PSF (for adherence studies) or TYI (1% FCS) (for amebic killing studies), then diluted in the final cell suspension solution to 1 mM. Amebas were exposed to 1 mM trypan blue for 1–24 h at 37°C before adherence to CHO cells or target cell killing was evaluated. Trypsin (Grand Island Biological Co.) was diluted to 0.125% in PSF. Amebas were incubated in trypsin (0.125%) for 15 min at 37°C, iced, and immediately used for studies of adherence (in PSF with 0.125% trypsin) or killing in TYI (1% FCS, 0.125% trypsin).

Studies of carbohydrate inhibition of amebic adherence and killing of target CHO cells. The following sugars and amino sugars (all Sigma Chemical Co.) were evaluated: N-acetyl-D-galactosamine (GALNAc), N-acetyl mannoside, N-acetyl glucosamine, α-D (+) fucose, D (+) galactose, D (+) xylose, maltose (hydrate), and neuraminic acid. Other sugars included D-mannose (Difco Laboratory, Detroit, Mich.), dextrose (Fisher Scientific Co., Pittsburgh, Pa.), chitotriose (kindly provided by D. Kobiler and D. Mirelman, Weizman Institute, Rehovoth, Israel) and GALNAc (Pfanstiehl Laboratory, Inc., Waukegan, Ill.). The solutions with the carbohydrate, 2×10^5 CHO cells, and 1×10^4 amebas were maintained at 4°C until start of the study. Studies of adherence were done in PSF, and studies of killing were done in TYI with 1% FCS. In studies of amebic destruction of CHO cell monolayers, 1×10^4 amebas were added to confluent CHO cell monolayers in TYI with 1% FCS in Linbro tissue culture plates (3).

^{111}InOx label was used as described previously: corrected specific killing

$$= \frac{\% \text{ supernatant counts (Eh − control)}}{80 − \% \text{ supernatant count of control}} \times 100;$$

where 80% is the maximal ^{111}InOx release (3, 13).

Dextran suspension studies. Following incubation of pellets in TYI (1% FCS) for 1 h at 4°C, amebas (with adherent CHO cells) and CHO cells were suspended in a 10% solution of Dextran (500,000 mol wt, Sigma Chemical Co.) in TYI (1% FCS). Suspensions were incubated at 37°C for the desired time, and CHO cell viability was studied by trypan blue exclusion as described above. Cyto B, Cyto D, and GALNAc were added at desired concentrations to the Dextran solution.

Statistics. All results are expressed as the mean±1SEM. Unpaired Student's t tests were used to evaluate differences.

RESULTS

Temperature sensitivity of target cell killing by Eh. To study adherence of CHO cells to amebas it was necessary to inhibit the killing and phagocytosis of target CHO cells. We found that reduced temperature inhibited both killing and phagocytosis of CHO cells by *E. histolytica*. We observed increasing target cell survival as determined by trypan blue exclusion as the incubation temperature was lowered to 31° and 25°C, and CHO cell survival at 4°C equaled controls without amebas (Table I). The number of trypan blue-positive CHO cells decreased to values not significantly different from controls (Table I) at 31° and 25°C ($P < 0.0005$ vs. 37°C), probably indicating decreased killing of target CHO cells at the amebic sur-

TABLE I

Inhibition of Amebic Killing of CHO Cells at 2 h by Reduced Temperatures
(Expressed as Percentages of the Original Number of CHO Cells)

	Temperature			
	37°C	31°C	25°C	4°C
Intact CHO cells				
Controls (CHO alone)	93.8±6.3	91.4±6.7	85.9±5.7	101.5±7.5
	($n = 16$)	($n = 8$)	($n = 11$)	($n = 6$)
CHO cells and amebas*	12.2±2.8	51.6±5.9	66.9±5.5	98.0±7.0
	($n = 18$)	($n = 9$)	($n = 11$)	($n = 6$)
Trypan blue (+) CHO cells				
Control	7.1±1.3	3.1±0.9	8.5±1.7	2.0±0.8
	($n = 16$)	($n = 8$)	($n = 11$)	($n = 6$)
CHO cells and amebas	32.5±3.3‡	8.9±1.6	10.1±2.4	6.5±0.6
	($n = 18$)	($n = 9$)	($n = 11$)	($n = 6$)

* Each successive decrease in temperature was associated with a significant increase in the percentage of CHO cells that were intact ($P < 0.05$), to 4°C at which no significant amebic killing occurred.
‡ Significantly greater than controls or colder temperatures ($P < 0.01$).

TABLE II
Inhibition of ^{111}InOx Release from Target CHO Cells at 2 h by Reduced Temperature (Expressed as Percentages of Total ^{111}InOx Released

	Temperature	
	37°C	25°C
Control (CHO cells alone)	3.8±0.2 (n = 6)	3.1±0.2 (n = 6)
CHO cells + amebas	38.4±4.1* (n = 6)	5.4±0.2 (n = 6)

* Different from CHO alone and from 25°C (P < 0.01 in both instances).

face. The increase in viable CHO cells seen at 31°–4°C would therefore represent decreased phagocytosis of target cells by amebas. This was confirmed in studies using ^{111}InOx-labeled CHO cells: release of label from target CHO cells after 2 h with amebas at 25°C did not differ from a control of CHO cells alone (Table II). The disappearance of target CHO cells without release of ^{111}InOx suggests that intact CHO cells are ingested by amebas at 25°C. In contrast, 38.4% of label is released when amebas are added at 37°C (Table II).

Adherence of amebas to target CHO cells. Inhibition of killing and phagocytosis of target cells by amebas at 4°C allowed us to examine Eh adherence to CHO cells with the formation of ameba-CHO cell rosettes (Fig. 1). Immediately after centrifugation, 34.2±5.0% of amebas formed rosettes with CHO cells; ameba-CHO cell rosettes increased to a maxi-

mum of 78.9±2.6% of amebas after 2 h of incubation at 4°C (P < 0.0005). Upon warming ameba-CHO cell rosettes to 37°C, the adherent CHO cells were moved to one area of the amebic surface (Fig. 1).

When amebas were incubated with CHO cells at 37°C for 20 min following centrifugation no significant CHO cell death occurred (100.5±9.0% of CHO cells remained viable by trypan blue exclusion, n = 6). 67% of amebas had at least three adherent CHO cells aggregated on the uroid portion of the ameba.

Effect of cytochalasins on amebic adherence. We have previously shown that Cyto B and D, microfilament inhibitors (14), interfere with amebic contact-dependent killing of target cells (3, 4). When Eh and CHO cells were first incubated in Cyto D (10 μg/ml) at 37°C then cooled to 4°C, fewer amebas formed rosettes with target CHO cells (Table III). Adherence of amebas to CHO cells at 37°C is decreased by Cyto B and D. (5 μg/ml) and nearly abolished with 10 μg/ml of Cyto D (Table III).

Amebic adherence to and killing of target CHO cells in calcium- and magnesium-free media. Formation of ameba-CHO cell rosettes at 4°C was unaltered in media free of magnesium and calcium (<0.1 μM) with excess EDTA (10 mM) (76.5±4.4% vs. control of 78.0±4.7%, at 2 h, n = 10). Amebic killing of target CHO cells (determined by trypan blue exclusion at 37°C) was also unaltered in calcium- and magnesium-free medium (39.6±5.9% CHO cell survival vs. 47.22±7.6% in controls at 2-h incubation).

Adherence of Eh to HRBC, BRBC, and SRBC. Eh has been previously reported to phagocytize HRBC (7) and contain a hemagglutinating lectin (6). Eh
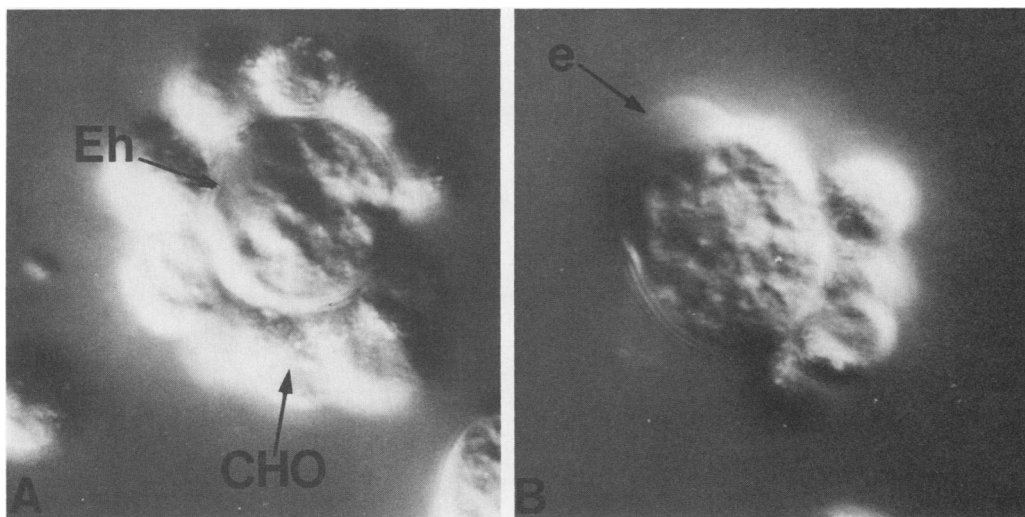

FIGURE 1 Amebas with adherent CHO cells at 4°C (A) and 37°C (B). At 37°C, adherent CHO cells are aggregated in one area of the ameba, away from advancing ectoplasm (e) which forms pseudopodia. Photomicrographs taken with a Zeiss Axiomax using interference contrast optics, ×1,000.

TABLE III

Inhibition of Amebic Adherence to CHO Cells by Cyto-
chalasins at 37° but Not 4°C (Expressed as Per-
centage of Amebas Forming Rosettes with
CHO Cells following Centrifugation
and Indicated Incubation)

	120 min at 4°C	15 min at 37°C
Control (Eh and CHO)	72.0±4.7 (n = 38)	83.3±3.6 (n = 6)
plus Cyto B (5 μg/ml)	80.2±3.8 (n = 8)	54.6±2.5* (n = 6)
Cyto D (5 μg/ml)	ND	14.8±3.3* (n = 6)
Cyto D (10 μg/ml)	71.0±8.5 (n = 6)	0.9±0.7* (n = 6)
	30 min at 4°C	
Control (Eh and CHO)	62.3±4.2 (n = 10)	
Eh exposed to Cyto D (10 μg/ml) at 37°C for 15 min, then centrifuged with CHO cells at 4°C	27.5±6.8‡ (n = 6)	

* Significantly decreased from control at 37°C and adherence with Cyto B (5 μg/ml) > Cyto D (5 μg/ml) > Cyto D (10 μg/ml) ($P < 0.001$).
‡ Significantly less than control ($P < 0.005$).

TABLE IV

Amebas Exhibit Greater Adherence to HRBC than BRBC
or SRBC (Expressed as Percentage of Amebas That Form
Rosettes with RBC after Centrifugation at 150 g
and 2-h Incubation at 4°C)

HRBC				
Type A	Type B	Type O	BRBC	SRBC
95.7±1.5 (n = 6)	97.6±1.9 (n = 6)	96.6±0.6 (n = 12)	40.4±5.9* (n = 12)	42.1±3.7* (n = 12)

* Significantly less than adherence of HRBC to Eh ($P < 0.001$).

posed to and remaining in trypsin (0.125%) were viable and retained their capacity to kill target CHO cells (58.7±5.7 CHO cell survival in trypsin vs. 53.0±8.5% with unexposed Eh, at 60 min, n = 5).

Carbohydrate inhibition of amebic adherence and killing of target CHO cells. Adherence of amebas to target CHO cells at 4°C (at incubation time of maximal rosette formation, 120 min) was significantly decreased by N-acetyl-D-galactosamine at concentrations as low as 0.0318 g/100 ml (1.44 mM, $P < 0.025$, Fig. 2). Of other carbohydrates tested, only D (+) galactose showed a significant inhibitory effect at millimolar concentrations, but with a lower level of inhibition than GALNAc ($P < 0.01$, Fig. 2). GALNAc also inhibited adherence of amebas to CHO cells at 37°C (67.7±4.8% of controls formed rosettes; vs. 12.1±5.7% formed rosettes with 1% GALNAc, $P < 0.001$; and 44.1±11.5% with 0.1% GalNAc, $P < 0.03$). The receptor for GALNAc appears to be on the ameba rather than CHO cell, as exposure of amebas to GALNAc at 4°C for 15 min (followed by washing) inhibited amebic rosette formation (by 29.5% in 15 paired studies, $P < 0.001$). The supernatant from this experiment was not inhibitory in immediate subsequent studies. Prior incubation of CHO cells with GALNAc (1%) did not inhibit the adherence of amebas to CHO cells (in 10 paired studies 68.9%±9.0 rosette formation after

formed rosettes equally well with all three ABO human blood groups (Table IV). Adherence of amebas to BRBC and SRBC was less than that observed for HRBC, demonstrating species specificity ($P < 0.005$, Table IV). Eh does not have Fc or complement receptors as no significant increase in rosette formation was observed with BRBC coated with IgG or IgM and SRBC coated with complement (Tables V).

Effect of trypan blue and trypsin on amebic adherence and killing of CHO cells. Trypan blue, at 1 mM, has been reported to inhibit leukocyte complement receptors (15) and to interfere with the actions of lysosomal enzymes (16, 17). Trypan blue (1 mM) did not alter either ameba-CHO cell rosette formation or amebic killing of target CHO cells (91.2±2.9% of trypan blue-exposed amebas formed rosettes with CHO cells at 4°C [n = 12], 30.2±8.0% CHO cell survival with trypan blue added vs. 27.4±6.9% with Eh alone [n = 11]).

Adherence of Eh to CHO cells was not sensitive to 0.125% trypsin (66.9±4.3% of amebas treated with trypsin-formed rosettes with CHO cells at 4°C vs. 65.2±5.7% in matched controls, n = 6). Amebas ex-

TABLE V

Lack of Fc or Complement Receptors on E. histolytica
(Expressed as Percentage of Ameba That Form
Rosettes with RBC after Centrifugation at
50 g Followed by Incubation
at 4°C for 2 h)

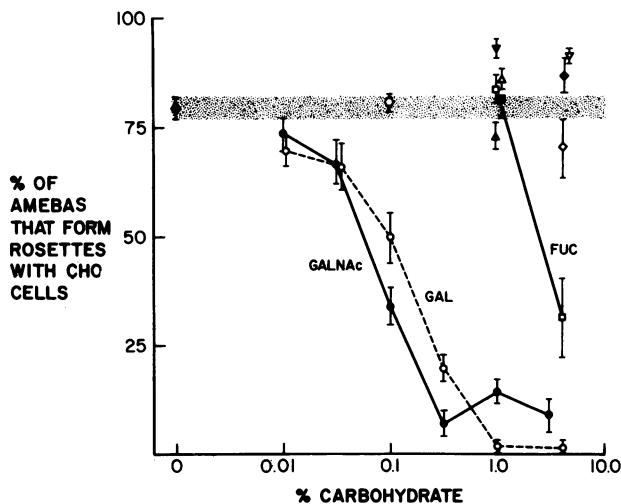|	Control	IgG-coated	IgM-coated
BRBC	0.2±0.2 (n = 5)	1.0±1.0 (n = 5)	12.4±8.9 (n = 5)
	Control	EAC coated	
SRBC	1.7±1.0 (n = 5)	1.2±1.0 (n = 5)	

FIGURE 2 Carbohydrate inhibition of amebic adherence to CHO cells at 4°C. N-acetyl galactosamine (●) significantly reduced amebic rosette formation at ≥0.0318 g/100 ml (bars represent means±SE of 6–17 studies) compared to control (●) (mean±SE of 38 studies, P < 0.025). Sigma Chemical Co.: N-acetyl galactosamine supplied by Pfanstiehl also inhibited ameba adherence to CHO cells at 0.318 and 0.1% (13.3±1.4 and 25.0±6.2% rosette formation, respectively, P < 0.005 compared with control). D (+) galactose (O) also inhibited amebic adherence to CHO cells at ≥0.0318% (bars are means±SE of 6–11 studies, P < 0.025). N-Acetyl galactosamine exhibited greater inhibition than D (+) fucose (□) which was only inhibitory at 4% (n = 6, P < 0.005). The following carbohydrates were noninhibitory (all means±SE of six studies): neuraminic acid 1% (▲), maltose 1% (■), n-acetyl glucosamine 4% (▽), n-acetyl mannoside 1% (▼), chitotriose 0.1% (□), D-xylose 1.0% (△), dextrose 4% (◇), and mannose 4% (◆).

GalNAc, and controls were 64.97±10.6%). Adherence of amebas to HRBC (type O) at 4°C was decreased 88% and 16.4% by GALNAc at 1.0 and 0.1%, respectively (P < 0.005).

GALNAc at concentrations of 4 and 1% also inhibited amebic killing of target CHO cells. This inhibition was observed both in amebic destruction of confluent CHO cell monolayers (Fig. 3a) and in ameba-CHO cell pellet studies (Fig. 3b). Glucose (at 1 and 4%) did not inhibit CHO cell killing (P < 0.025 vs. GALNAc). Studies of ^{111}InOx release from target CHO cells showed that GALNAc completely inhibited contact-dependent lysis of target CHO cells by Eh (Table VI). Yet, as shown in Fig. 3b, a significant number of CHO cells disappeared under these conditions, suggesting that they were phagocytized intact by amebas when protected by 4% GALNAc. The percentage of intact CHO cells decreased from 93.1% in controls to 63.8% with Eh in 4% GALNAc (P < 0.0005, Fig. 3b).

Studies using dextran suspension of Eh and CHO cells. As described by Martz (18), cells suspended in high molecular weight (500,000) dextran solutions
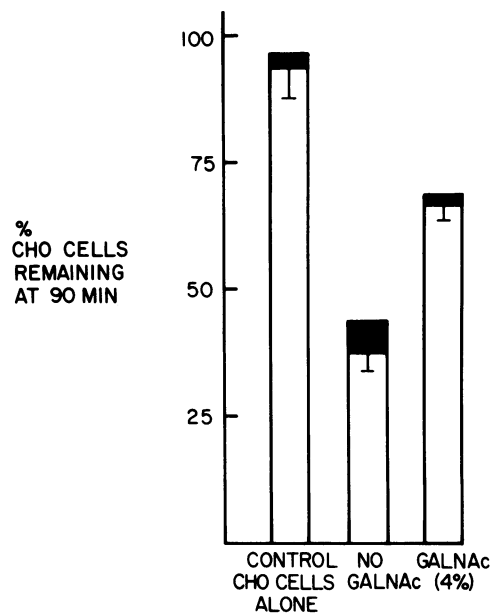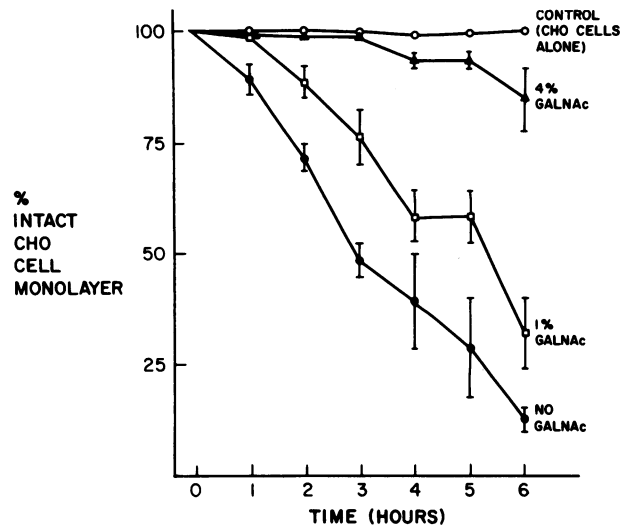

FIGURE 3 Inhibition of amebic destruction of CHO cell monolayers and cytolysis of individual CHO cells by N-acetyl galactosamine. (A) At ≥2 h, 1% GALNAc reduced amebic destruction of CHO cell monolayers and 4% GALNAc showed significantly greater inhibition (n = 6 paired studies, P < 0.01). (B) 4.0% GALNAc also reduced amebic killing of suspended and centrifuged CHO cells (P < 0.005); the number of trypan blue-positive CHO cells (cross-hatched areas) were decreased to control values (P < 0.005).

remain isolated from one another. Under these conditions, cell interactions can occur only if cells are adherent to one another before suspension in the dextran solution. When amebas and CHO cells were suspended in dextran, without prior centrifugation to allow for rosette formation, no CHO cell death occurred compared with controls with CHO cells alone

TABLE VI

TABLE VI

Inhibition of ^{111}Indium Ox Release from Target CHO Cells at 90 min by 4% GALNAc (Expressed as Both Percentage of Total ^{111}InOx That Is Released and Corrected Specific Killing)

	Control media	4% GALNAc
Control (CHO cells above)	9.8±0.8 (n = 11)	11.5±0.6 (n = 12)
CHO cells plus amebas	25.1±3.1* (n = 12)	11.1±1.5 (n = 12)
Corrected specific killing of CHO cells by amebas	21.9±5.5‡ (n = 11)	-0.35±1.6 (n = 12)

* Significantly different from control and with 4% GALNAc ($P < 0.005$).

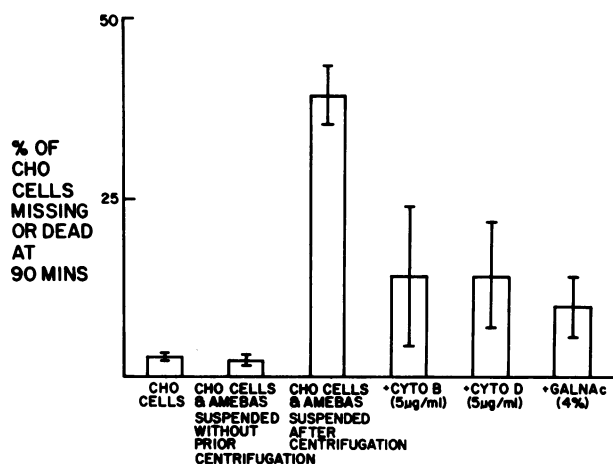‡ Significantly greater than with 4% GALNAc present ($P < 0.001$).

FIGURE 4 Dextran suspension of cells demonstrates that cytochalasins and GALNAc inhibit amebic killing of adherent CHO cells. When cells are suspended at 37°C without prior centrifugation in Dextran, no CHO cell death occurs compared with controls. However, significant CHO cell death occurs in dextran at 37°C after amebas and CHO cells are first centrifuged and incubated at 4°C to allow for adherence. Cyto B and D (5 μg/ml) and GALNAc (4%) reduced amebic killing of adherent CHO cells ($P < 0.025$ for Cyto, $P < 0.005$ for GALNAc).

(Fig. 4). This finding confirms that direct contact is required for amebas to kill target CHO cells. When amebas and CHO cells were centrifuged, incubated at 4°C for 1 h to allow for adherence, and the pellet was then suspended in dextran (at 37°C), amebas killed 36.2% of target CHO cellls ($P < 0.005$, Fig. 4). The percentage of amebas with adherent CHO cells was unaltered by suspension in dextran solutions. The percentage of amebas that formed rosettes in PSF at 1 h was 65.6±4.4% and was 71.1±5.1% after suspension of the pellet in 10% dextran for 1 h at 4°C. After 1 and 2 h at 37°C, 35.5±8.9 and 71.2±7.3% of CHO cells which were adherent to amebas were trypan blue positive (vs. 6.6±0.6 and 5.2±1.3% of nonadherent CHO cells at 1 and 2 h, respectively, $P < 0.01$).

Cyto B and D (5 μg/ml) in dextran inhibited amebic killing of adherent target CHO cells by 61.8 and 58.6%, respectively (Fig. 4). GALNAc (4%) also decreased amebic killing of previously adherent CHO cells when ameba-CHO rosettes (formed at 4°C) were suspended in dextran at 37°C (Fig. 4). GALNAc (4%) eluted CHO cells from Eh, whereas Cyto D did not. The percentage of Eh with adherent CHO cells (in TYI-dextran at 37°C) was reduced from 47.4±6.0 to 11.5±7.8% and 6.5±2.2% after 1 and 2 h, respectively, with GALNAc (4%) ($P < 0.0005$). No significant reduction occurred after incubation in Cyto D (5 μg/ml) for 2 h (36.7±7.5% of Eh with adherent CHO cells).

DISCUSSION

The ability of *E. histolytica* to kill target cells on direct contact is well recognized (1-6). In these studies we have utilized the inhibition of amebic killing by reduced temperatures to study a separate, prior adherence event which appears to be necessary before amebas can lyse cells. Both the adherence and the extraamebic cytolethal events can be separated from cytophagocytosis by amebas.

The temperature sensitivity of the amebic cytolytic capacity (which is abolished at 25°C) is consistent with a requirement for intact amebic microfilament function (3, 4). Actin gelatin studies in both *Dictostelium discoideum* and *Acanthamoeba* demonstrate the temperature dependence of actin polymerization and contraction (and thus microfilament function) (19, 20). Further reduction of temperature to 4°C also abolishes amebic phagocytosis, which allowed us to specifically study the adherence of amebas to target cells. Using a standard method to evaluate adherence (21), we found that amebas formed rosettes with target CHO cells at 4°C. At 37°C adherent CHO cells are aggregated on the uropoid area of *E. histolytica*, where concanavalin A and antiamebic antibody have been shown to aggregate (22).[2]

Cyto B and D, by inhibiting microfilament function (14), inhibit patching and capping of integral membrane protein receptors and alter cell shape (23), both of which could influence cell to cell adherence (24). Although ineffective at 4°C (which may preserve receptor "patches"), cytochalasins at 37°C have an

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[2] Ravdin, J. I., D. L. Weinbaum, H. D. Kay, and R. L. Guerrant. Studies of the aggregation and nature of target-cell and Con A receptors on *E. histolytica*. Presented at the Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, Ga. 7 November 1980. Paper 174.

inhibitory effect on amebic adherence that persists when cytochalasin-exposed amebas are cooled to 4°C. These findings suggest that adherence of amebas to cells involves receptors that must be actively maintained in an effective conformation. Cytochalasins also interfere with adherence events of other effector cells such as human lymphocytes and macrophages (25, 26). In contrast to lymphocytes or numerous toxins which require divalent cations to kill target cells (27, 28), we found no reduction in amebic adherence to or killing of target cells in media with ≤0.1 μM calcium and magnesium plus excess EDTA. However, we cannot exclude a role for local release of divalent cations from intracellular compartments within the cellular pellet (18).

Initial investigations into the nature of the amebic receptor for CHO cells revealed it to be trypsin resistant and unaltered by trypan blue (which blocks neutrophil complement receptors [15]). Consistent with these studies, we found that amebas do not bind to antibody or cells via Fc or complement receptors. Amebas adhered equally well to A, B, and O group HRBC and had greater adherence to human erythrocytes than BRBC or SRBC.

The adherence of amebas to target CHO cells demonstrates carbohydrate specificity and is a required component of the contact-mediated killing of target cells. The specificity of this adherence event and its apparent analogy with lectin binding is demonstrated by its inhibition with N-acetyl-D-galactosamine, and to lesser extent by D-galactose. Furthermore, inhibition of amebic adherence prevents destruction of either isolated or monolayers of CHO cells, thus providing evidence that this adherence event is the one involved in the subsequent amebic cytolethal process. Indirect evidence suggests that amebic phagocytosis continued despite the presence of N-acetyl-D-galactosamine, indicating that the adherence event we studied is not required for amebic phagocytosis. Osmotic effects may be important (29), but would not explain the carbohydrate specificity observed in our model. Carbohydrate-induced changes in amebic metabolism would not alter amebic adherence at 4°C (24). Similar amebic receptors appear to be involved in adherence to CHO cells and HRBC, as N-acetyl-D-galactosamine also inhibited adherence of amebas to HRBC.

The importance of amebic adherence has been emphasized morphologic with in vivo and in vitro studies which demonstrated amebas adherent to guinea pig intestinal mucosa prior to penetration (30, 31). The hemagglutinating lectin isolated by Kobiler and Mirelman (8) from E. histolytica may not be involved in the adherence of whole viable amebas to target cells, as chitotriose (at ~50 times the 50% lectin inhibitory concentration [8]) did not decrease amebic adherence to CHO cells. Mattern et al. (32)

have reported inhibition of the nonlethal cell rounding effect of a "lectinlike" amebic extract with serum, fetuin, and high concentrations of N-acetyl-D-galactosamine (100–400 nM or ~2–8%).

Using the dextran suspension method as described by Martz (18), we were able to study separately the amebic killing step which follows adherence. This method also confirms our previous reports (3, 4) of the need for direct amebic contact for E. histolytica to kill target cells. Cyto B and D, which at 37°C inhibit amebic adherence, also interfere with the killing of target cells by amebas after adherence has occurred. As expected, N-acetyl-D-galactosamine was able to elute CHO cells from amebas and thus decrease amebic killing of previously adherent CHO cells.

The mechanism of target cell death following adherence, although requiring intact amebic microfilament function, remains unknown. The lysosomal enzyme inhibitor, trypan blue (16, 17), did not alter amebic killing of target CHO cells. This provides additional evidence that a surface-active lysosome (33) is not responsible for amebic killing following adherence. Amebic killing does not appear to involve oxidative mechanisms, be modulated by cyclic nucleotides, or be blocked by protease inhibitors (4).[3]

The techniques to evaluate the separate steps in the killing of target cells by amebas and the findings that N-acetyl-D-galactosamine prevents amebic adherence should now enable studies to determine the role of adherence and target cell killing in the pathogenesis of amebiasis.

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[3] Ravdin, J. I., and R. L. Guerrant. Adherence of cells to E. histolytica: distinction from temperature and microfilament-dependent killing. Presented at the International Congress of Antimicrobial Agents and Chemotherapy, New Orleans, La., 22 September 1980. Paper 8.

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