INFECTION AND IMMUNITY, July 1989, p. 2179–2186
0019-9567/89/072179-08$02.00/0

Vol. 57, No. 7

Characterization of Cell Surface Carbohydrate Receptors for *Entamoeba histolytica* Adherence Lectin

JONATHAN I. RAVDIN,[1,2,3,4]* PAMELA STANLEY,[5] CHERYL F. MURPHY,[1] AND WILLIAM A. PETRI, JR.[2,3,6]

Divisions of Clinical Pharmacology,[1] Geographic Medicine,[2] and Infectious Diseases,[3] Department of Medicine, Box 419, and Departments of Pharmacology[4] and Microbiology,[6] University of Virginia School of Medicine, Charlottesville, Virginia 22908, and Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461[5]

Received 15 December 1988/Accepted 30 March 1989

Binding and cytolysis of Chinese hamster ovary (CHO) cells by *Entamoeba histolytica* trophozoites is inhibitable by galactose (Gal) or *N*-acetyl-D-galactosamine (GalNAc). To better define the carbohydrate receptor for *E. histolytica*, we compared the binding and cytolytic target properties of 10 CHO glycosylation mutants. Each mutant expresses a uniquely altered array of N- and/or O-linked cell surface carbohydrates. Amebic adherence was reduced when lactosamine-containing N-linked carbohydrates were essentially absent (Lec1 mutant), almost undetectable when Gal and GalNAc residues were absent on both N- and O-linked carbohydrates (ldlD.Lec1 mutant), and enhanced for mutants with increased terminal Gal residues (Lec2 and Lec3). Parental CHO cells treated with neuraminidase to expose Gal residues behaved like Lec2 mutants. Binding of purified Gal or GalNAc lectin to parental, Lec1, ldlD.Lec1, and Lec2 mutant CHO cells corroborated the adherence results. The suitability of CHO cell mutants as targets for amebic cytolysis correlated with their glycosylation phenotype: the Lec1 mutants were less susceptible than parental CHO cells, the ldlD.Lec1 mutants were highly resistant, and the Lec2 mutants required higher concentrations of Gal for inhibition. The *E. histolytica* Gal or GalNAc adherence lectin bound preferentially to β1-6-branched, N-linked carbohydrates lacking terminal sialic acid or fucose residues. However, amebic lectin binding to either N- or O-linked cell surface carbohydrates was sufficient to initiate parasite cytolytic activity.

The enteric protozoan *Entamoeba histolytica* infects 10% of the world's population and is an important cause of morbidity and mortality due to resultant colitis and liver abscess (41). The pathogenesis of invasive amebiasis apparently involves attachment of trophozoites to the colonic mucus layer, mucus depletion, proteolytic disruption of mucosal barriers, and amebic adherence to and cytolysis of host epithelial and inflammatory cells (2, 3, 11, 19, 39).

Using in vitro systems to study *E. histolytica* adherence and cytolytic mechanisms, we have described an amebic galactose (Gal)- and *N*-acetyl-D-galactosamine (GalNAc)-inhibitable adherence lectin (Gal-GalNAc lectin) that exclusively mediates amebic binding to target Chinese hamster ovary (CHO) cells (3, 18, 21, 22). Binding by the Gal-GalNAc lectin has been implicated in amebic cytolysis of CHO cells because Gal or GalNAc monomers inhibit *E. histolytica*-induced release of [^{111}In]indium oxine from labeled CHO cells, CHO cell monolayer destruction, and killing of CHO cells as determined by trypan blue exclusion (20, 21, 24). In addition, amebic cytolysis of human neutrophils, macrophages, and lymphocytes is also Gal-GalNAc inhibitable (24, 29, 30).

The *E. histolytica* Gal-GalNAc lectin, isolated by use of carbohydrate affinity chromatography and lectin-specific monoclonal antibodies (18), consists of a single 170-kilodalton heavy binding subunit linked by disulfide bonds to a light 35-kilodalton subunit of as yet unknown function (16b). Asialofetuin and asialoorosomucoid, having 9 to 16 β-1-4-linked terminal Gal residues, are 1,000-fold more effective than Gal monomers in inhibiting amebic adherence to CHO cells (18), suggesting that the cell surface carbohydrate receptor recognized by the amebic adherence lectin has a complex structure. Purified colonic mucins, a heterogenous group of molecules, have a complex carbohydrate structure rich in Gal and GalNAc (16, 31); at 4°C colonic mucins are high-affinity receptors for the *E. histolytica* Gal-GalNAc lectin (3, 4, 25). This finding provides the biochemical basis for *E. histolytica* intestinal colonization and further supports the physiologic relevance of characterizing the binding of cell-associated and purified Gal-GalNAc lectin to complex CHO cell surface carbohydrates.

Knowledge of the structure of the target cell carbohydrate receptor for the *E. histolytica* Gal-GalNAc lectin is necessary to fully understand *E. histolytica* adherence and cytolytic activities and thus pathogenesis of invasive amebiasis. In a recent study, Li et al. (13) provided evidence that lactosamine units on N-linked carbohydrates are the major receptors for *E. histolytica* adherence. However, lactosamine units exist on many different types of N-linked carbohydrates and, depending on their molecular environment, differ profoundly in their ability to bind specific lectins (8). In this paper we provide evidence that lactosamine units on β-1,6-branched, N-linked carbohydrates deficient in sialic acid are the best receptors for the *E. histolytica* Gal-GalNAc lectin. However, only when Gal and GalNAc residues were missing from both N- and O-linked carbohydrates was a high degree of resistance to amebic cytolysis observed. Therefore, both N- and O-linked carbohydrates may serve as receptors for initiation of invasive amebiasis in vivo.

MATERIALS AND METHODS

Parasite and CHO cell cultivation. Axenic *E. histolytica* trophozoites, strain HM1:IMSS (ATCC 30459; American Type Culture Collection, Rockville, Md.), were originally obtained from L. S. Diamond (National Institutes of Health, Bethesda, Md.). Amebae have been maintained in our laboratory for 6 years, passed through gerbil liver, and cloned in semisolid agar (designated HM1:IMSS, clone 7). All amebae

* Corresponding author.

were grown by the method of Diamond et al. (7) in TYI-S-33 (Trypticase [BBL Microbiology Systems, Cockeysville, Md.], yeast extract, iron, and serum) medium supplemented with 100 U of penicillin (GIBCO Laboratories, Grand Island, N.Y.) per ml and 100 μg of streptomycin sulfate (GIBCO) per ml; trophozoites were harvested as described previously (20).

CHO cells cultivated included a Gat^-2 parent and Lec1, Lec2, Lec3, Lec4, Lec8, Lec9, LEC10, LEC11, LEC12, and ldlD.Lec1 glycosylation mutants (28, 34–38). The specific clones used were as follows: Gat^-Lec1.1N, Pro^-Lec2.6A, Pro^-Lec3.4B, Pro^-Lec4.7B, Gat^-Lec8.1C, Pro^-Lec9.12A, Pro^-LEC10.3C, Pro^-LEC11.E7, Pro^-LEC12.1B, and Pro^-ldlD.Lec1.1.5. CHO cells were grown in MEM alpha medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml). To ensure maximal inhibition of O-linked carbohydrate synthesis (M. Krieger, P. Reddy, K. F. Kozarsky, D. M. Kingsley, and M. Penman, Methods Cell Biol., in press), ldlD.Lec1 cells were grown for 3 days before study in serum-free Ham F-12 medium (GIBCO) containing 5 μg of insulin per ml, 5 μg of transferrin per ml, and 5 ng of selenious acid (all from Collaborative Research, Inc., Bedford, Mass.) per ml. Cells were harvested by trypsinization (0.25%) and suspended in medium 199 (GIBCO) supplemented with 5.7 mM cysteine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 0.5% bovine serum albumin (M199S) with 1% fetal bovine serum.

Studies of adherence of *E. histolytica* trophozoites to target CHO cells. Adherence studies were performed by using a rosetting assay as described previously (21); amebae (1×10^4) and CHO cells (2×10^5) were suspended in 1 ml of M199S without serum, centrifuged at $150 \times g$ for 5 min, and incubated for 2 h at 4°C. After incubation, 0.8 ml of the supernatant was removed, and the pellet was suspended by vortexing the tube for 5 s. One drop of the cell suspension was placed on the hemacytometer chamber, and the cover slip was applied. The numbers of amebae that formed rosettes with CHO cells (three or more CHO cells adherent to each ameba) were counted (≥50 amebae were counted per chamber); studies were conducted with and without Gal (10 mg/ml) or GalNAc (10 mg/ml).

Neuraminidase treatment of target CHO cells. Gat^-2 parent CHO cells were harvested by trypsinization, washed, and placed in MEM alpha medium with 10% fetal bovine serum (pH 5.5) at 10^7 cells per ml. CHO cells were incubated with and without neuraminidase (1 U of type V from *Clostridium perfringens*; Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C (37). Cells were washed, incubated for 15 min at 4°C in MEM alpha medium with 10% fetal bovine serum, and washed twice in saline (Dulbecco phosphate-buffered saline; GIBCO) before being used in adherence or lectin-binding assays.

Purification of the *E. histolytica* Gal-GalNAc lectin by monoclonal antibody affinity chromatography. Trophozoites harvested from 72-h cultures were incubated 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 anti-lectin monoclonal antibodies H8-5, 7F-4, 5B-8, 3F-4, and 6D-2 (18) per ml immobilized on 5 ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) by the method of Petri et al. (16a, 17). The supernatant was recirculated through the column with a peristaltic pump overnight at 4°C. The column was then extensively washed with solubilization buffer, first with and then without Nonidet P-40. The bound amebic lectin was eluted with 0.2 N acetic acid (pH 2.5), which was immediately neutralized by collection into 5 ml of 2 M Tris buffer (pH 8.2). Adherence lectin was concentrated by centrifugation in Centricon 10 microconcentration devices (Amicon Corp., Danvers, Mass.), and protein concentration was determined by the Pierce BCA method as recommended by the manufacturer. Successful purification for each batch of lectin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining demonstrating the characteristic 170- and 35-kilodalton lectin subunits, which by laser densitometry has indicated 70% purity of the lectin (16b). The adherence lectin is purified 500-fold by this one-step immunoaffinity chromatography as judged by a radioimmunoassay (16b). Lectin was stored at −70°C before use.

Determination of the binding of purified *E. histolytica* Gal-GalNAc lectin to CHO cells. CHO cells (5×10^5) were incubated with immunoaffinity-purified Gal-GalNAc lectin (1.0 μg/ml) with or without Gal (20 μg/ml) for 60 min at 4°C in phosphate-buffered saline. After cells were washed, they were incubated with 500,000 cpm of iodinated anti-lectin monoclonal antibody 7F-4 (16a) per 100 μl for 60 min at 4°C. The cells were extensively washed, and bound ^{125}I activity was determined in a gamma counter. Data are expressed as Gal-inhibitable ^{125}I counts bound, which equals [^{125}I counts bound $_{(lectin)}$ − ^{125}I counts bound $_{(control)}$] − [^{125}I counts bound $_{(lectin + Gal)}$ − ^{125}I counts bound $_{(control + Gal)}$].

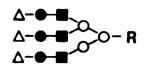
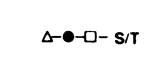
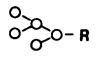
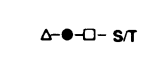
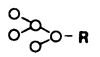
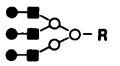
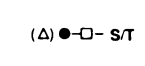
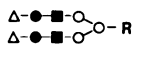
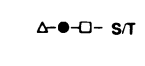
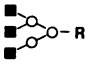
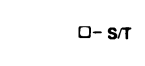
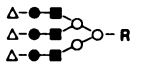
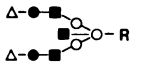
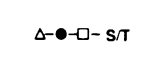
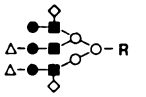
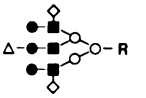
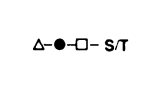
Determination of amebic extracellular cytolysis of target CHO cells. Harvested CHO cells (2×10^5) with and without amebae (10^4) were placed in M199S with 10% fetal bovine serum (1.0 ml), centrifuged at $150 \times g$ for 5 min, and incubated for 2 or 3 h at 37°C with or without Gal. For the cell sorter studies, after incubation, the cells were placed on ice, Gal was added to all tubes for a minimal final concentration of 10 mg/ml, and the tubes were vortexed for 5 s to elute adherent CHO cells from *E. histolytica* trophozoites (21). Amebae, viable CHO cells, and dead but still intact CHO cells (10^6 CHO cells per ml) were identified by size and density and quantified in a cell sorter. In the cell sorter plots, forward angle light scatter was proportional to cell size (*x* axis), the logarithm (base 10) of the integral of the 90° light scatter was proportional to cell density (*y* axis), and cell number was quantified on the *z* axis. Each experiment was the result of a pool of six separate samples; the percentage of viable and dead CHO cells was calculated on the basis of the number of cells placed in the tubes. Some loss of CHO cells occurred during cell sorting, so the percentages did not add up to 100. Amebic cytolysis of target CHO cells was also determined by hemacytometer counts of viable CHO cells by using trypan blue exclusion as reported previously (20, 21).

Statistics. Data are expressed as the mean ± one standard error of the mean. Significance was determined by unpaired or paired Student *t* test as appropriate.

RESULTS

Adherence of *E. histolytica* trophozoites to CHO glycosylation mutants. The carbohydrate structures shown in Table 1 are representative of typical N- and O-linked moieties predicted to be present at the membrane of CHO cells and the glycosylation mutants on the basis of previous carbohydrate

TABLE 1. Adherence of axenic *E. histolytica* trophozoites to CHO cells at 4°C

CHO Cell Line	Carbohydrates[a]		% of amebae forming a rosette with CHO cells[b]	
	N-linked	O-linked	Control	Galactose (10 mg/ml)[c]
Parent Gat⁻2			65.2 ± 1.6 n = 12	6.6 ± 1.4 n = 12
Lec1			11.5 ± 0.9[d] n = 12	4.3 ± 0.8 n = 12
ldl D. Lec1			2.7 ± 0.7[e] n = 12	1.3 ± 0.3 n = 12
Lec2			91.9 ± 1.1[f] n = 10	26.8 ± 2.8[f] n = 10
Lec3			86.9 ± 1.0[f] n = 10	20.8 ± 2.6[g] n = 10
Lec4			49.5 ± 1.9 n = 10	5.7 ± 0.6 n = 10
Lec8			43.0 ± 1.6 n = 12	3.6 ± 0.9 n = 12
Lec9			66.4 ± 3.9 n = 10	0.8 ± 0.4 n = 10
LEC10			69.2 ± 3.6 n = 10	4.0 ± 1.0 n = 10
LEC11			75.2 ± 2.1 n = 10	6.2 ± 0.7 n = 10
LEC12			35.5 ± 1.7 n = 10	5.19 ± 0.7 n = 10

[a] Symbols: △, sialic acid; ●, Gal; ■, *N*-acetylglucosamine; ○, mannose; □, GalNAc; ◇, fucose; R, GlcNAc-β-1,4-GlcNAcβ1, Asn; S/T, serine or threonine.
[b] Amebae and CHO cells (1:20 ratio) were centrifuged at 150 × *g*, incubated in 1.0 ml at 4°C for 120 min, and vortexed for 5 s in 0.2 ml. Trophozoites need ≥3 adherent CHO cells to be designated a rosette; data are expressed as means ± standard errors of the means.
[c] For all results $P \leq 0.01$ compared with control medium.
[d] $P \leq 0.01$ compared with Gat⁻2 and all other mutants studied.
[e] $P \leq 0.01$ compared with Lec1 and not different from ldlD.Lec1 with galactose.
[f] $P \leq 0.01$ compared with Gat⁻2, and Lec4, Lec5, Lec8, Lec9, LEC10, LEC11, and LEC12.
[g] $P \leq 0.01$ compared with Gal present with Gat⁻2, Lec1, Lec3, Lec4, Lec8, Lec9, LEC10, LEC11, and LEC12.

structural and enzymic studies, which identified the basis for the glycosylation change in each mutant (1, 5, 6, 9, 12, 26–28, 32–38; Krieger et al., in press). The N-linked carbohydrates in parental CHO cells would be expected to contain up to three or more lactosamine units (Gal β-1,4-GlcNAc) (14). The most complex structure, that of the LEC11 mutant, is illustrated in Fig. 1. Little is known of the O-linked carbohydrates synthesized by CHO cells, so we hypothesized a very simple structure that occurs ubiquitously. The predicted truncations for the different mutants are based on the nature of their glycosylation defects, which reduce the availability or synthesis of a specific nucleotide sugar (5, 6, 12). Under serum-free growth conditions the ldlD mutation should be maximally expressed and allow little if any O-linked carbohydrate biosynthesis (Krieger et al., in press).

Amebic adherence to a parental CHO cell line, Gat⁻2, was 65% as measured by a rosette assay (Table 1). The number of rosettes was reduced to 6.6% by the presence of 10 mg of Gal per ml ($P < 0.01$). The results of amebic adherence with and without Gal (10 mg/ml) for 10 CHO glycosylation mutants are summarized in Table 1. Adherence to the Lec1 CHO cell mutant, which expresses Man_5 oligomannosyl rather than lactosamine-containing N-linked carbohydrates (27), was reduced to 12% but continued to be Gal inhibitable. Amebic adherence to the ldlD.Lec1 mutants, which possess Man_5 oligomannosyl N-linked carbohydrate structures and, under serum-free growth conditions, essentially no O-linked carbohydrate structures (36; Krieger et al., in press), was almost undetectable. Parasite adherence to Lec2 and Lec3 mutants, which express increased numbers of terminal Gal residues due to their sialylation defects (6,27), was markedly increased and less inhibited by 10 mg of Gal per ml. Amebic adherence was decreased for the following mutants: Lec8 CHO cells, which express increased numbers of terminal

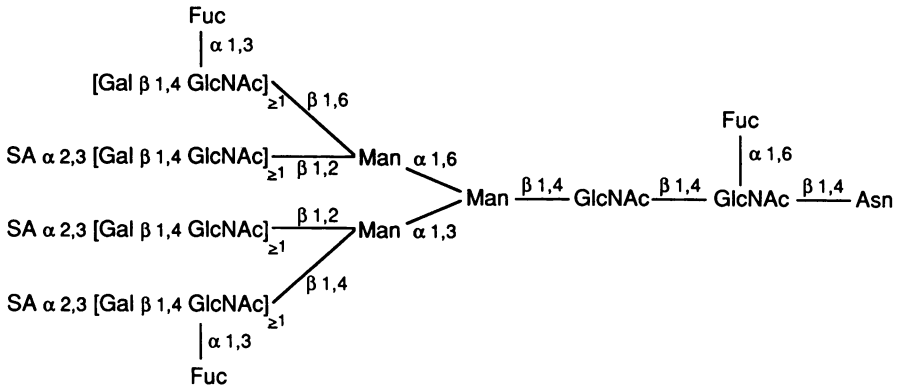


FIG. 1. The most complicated type of N-linked carbohydrate structure that can be predicted to occur from present knowledge would be on LEC11 glycoproteins as presented above. Abbreviations: Asn, asparagine; Fuc, fucose; Man, mannose; SA, sialic acid. Although it is known that CHO cell surface carbohydrates are mostly branched, the predominant species is not necessarily the β-1,6-branched triantennary structure shown here and would include β-1,4 triantennary and tetraanternnary moieties.

N-linked GlcNAc and O-linked GalNAc carbohydrates due to their galactosylation defect (5); Lec4 mutants, which lack β-1-6 branches on N-linked carbohydrates (38); and LEC12 mutants, which have α-1-3 linked fucose added to nonsialylated lactosamines (9). However, the presence of α-1-3 fucose on sialylated as well as nonsialylated lactosamines, as expressed at the LEC11 cell surface (9), correlated with a small increase in amebic adherence. Interestingly, amebic adherence was not apparently affected by the increased branching of N-linked carbohydrates that is characteristic of Lec9 and LEC10 mutants (Table 1). Lec9 mutants synthesize an increased proportion of β-1,6-branched, N-linked carbohydrates (26, 28), and LEC10 mutants add the bisecting GlcNAc to N-linked carbohydrates (1). It is of note that amebic adherence to Lec9 CHO cells was extremely sensitive to inhibition by Gal (98.8% inhibition).

Because adherence to Lec2 and Lec3 mutants was less sensitive to inhibition by Gal in comparison with the Gat$^-$2 parent (Table 1), it was of interest to determine the inhibitory effect of GalNAc, which is exposed on the O-linked carbohydrates of these mutants (Table 1). GalNAc at 10 mg/ml was not as effective as Gal in inhibiting amebic adherence to either parental or Lec2 or Lec3 mutant cells ($P < 0.01$ for each). Although Gal at 50 mg/ml almost completely inhibited amebic adherence to Lec2 mutants (96.1% inhibition), the addition of GalNAc and Gal (each at 10 mg/ml) did not increase the degree of inhibition above that observed with Gal alone for amebic adherence to parental, Lec2, or Lec3 CHO cells ($n = 6$ for each). We also studied adherence by using Gat$^-$2 parent CHO cells that had their terminal sialic acid residues enzymatically removed. Adherence of amebae to desialylated Gat$^-$2 cells increased to 89%, compared with 59.7% adherence to control Gat$^-$2 cells ($P < 0.01$). With Gal (10 mg/ml) present, amebic adherence was reduced to 27.9% for desialylated Gat$^-$2 cells but to 4.0% for control Gat$^-$2 cells ($P < 0.01$).

To approximate more physiologic conditions, amebic adherence to mutant CHO cells was also studied at 37°C (Table 2). Trophozoite adherence to the Gat$^-$2 parent was inhibited by 92% in the presence of Gal (10 mg/ml). Adherence to the Lec1 mutant was diminished in comparison with that of the parent CHO cell line, but less so than at 4°C, and remained Gal inhibitable. However, as observed at 4°C, amebic adherence to the ldlD.Lec1 mutant was barely detectable. Interestingly, at 37°C rosette formation with Lec2 mutants was no greater than that with parental CHO; however, as observed at 4°C, higher concentrations of Gal were required for inhibition of adherence (Table 2).

Binding of the purified *E. histolytica* Gal-GalNAc lectin to CHO cells. To directly measure binding of the *E. histolytica* Gal-GalNAc lectin to CHO cells, we developed a radioimmunoassay with immunoaffinity-purified adherence lectin and an iodinated anti-lectin monoclonal antibody. In complete agreement with the ameba-CHO cell adherence studies, Gal-inhibitable binding of the adherence lectin at 4°C was greatest to the Lec2 mutant (4,474 ± 428 cpm) and diminished for the Lec1 mutant (440 ± 46 cpm) in comparison with that of the parent Gat$^-$2 cell line (3,042 ± 178 cpm; $P < 0.01$ compared with Lec1 and $P = 0.027$ compared with Lec2). Gal-inhibitable adherence lectin binding was virtually absent for the N- and O-linked carbohydrate-deficient ldlD.Lec1 mutant (−10 ± 28 cpm; $P < 0.01$ compared with the Lec1 mutant).

Cytolysis of target CHO cells by *E. histolytica* trophozoites. To directly measure amebic contact-dependent cytolysis of CHO cells, a new assay in which the cell sorter was used to separate and quantitate viable and dead CHO cells was developed. After centrifugation and incubation of amebae and Gat$^-$2 CHO cells for 2 h at 37°C, there were two populations of CHO cells separable on the basis of cell size and density: viable target cells, which excluded trypan blue, and dead but still intact target cells, which took up trypan blue and had not yet been ingested by trophozoites or undergone complete dissolution. In a representative study (Fig. 2), there was marked amebic cytolysis of Gat$^-$2 cells

TABLE 2. Adherence of *E. histolytica* trophozoites to CHO cells at 37°C

CHO cell line	% of amebae forming a rosette with CHO cells	
	Control medium	Gal (10 mg/ml)[a]
Parent Gat$^-$2	48.5 ± 8.0	4.0 ± 1.3[b]
Lec1	28.9 ± 3.8[c]	1.7 ± 0.5[b]
ldlD.Lec1	2.2 ± 0.6[d]	1.0 ± 0.7
Lec2	40.0 ± 2.4	32.3 ± 3.9

[a] With 40 mg of Gal per ml, 5.3% of Lec2 amebae formed rosettes ($P < 0.01$).
[b] $P \leq 0.01$ compared with control medium.
[c] $P \leq 0.05$ compared with parent Gat$^-$2.
[d] $P \leq 0.01$ compared with any other cell line.

TABLE 3. Viability of target CHO cells during incubation with *E. histolytica* trophozoites

CHO cell line	% CHO cell viability after a 3-h incubation with amebae[a]	
	Control medium	Gal (10 mg/ml)
Parent Gat$^-$2	40.0 ± 5.8	82.8 ± 4.6[b]
Lec1	52.6 ± 6.5	101.5 ± 5.1[b]
ldlD.Lec1	86.1 ± 4.7[c]	94.4 ± 5.8
Lec2	42.1 ± 4.7	47.8 ± 2.9[d]

[a] Expressed as a percentage of viability in controls of CHO cells incubated under identical conditions without amebae present. With 40 mg of Gal per ml, the percent viability was 86.2% ($P < 0.01$).

[b] $P \leq 0.01$ compared with control medium.

[c] $P \leq 0.01$ compared with parent Gat$^-$2, Lec1, or Lec2 and not significantly reduced from control viability with Gal.

[d] $P \leq 0.01$ compared with parent Gat$^-$2 or Lec1 with Gal.

(2B, 32.5% viable and 43.3% dead, compared with 2A, 81.8% viable and 3.2% dead but intact). Gal markedly inhibited amebic cytolysis of Gat$^-$2 cells (2C, 62.0% viable and 16.7% dead). Amebae were able to deliver a lethal hit to the Lec1 mutant (2E, 39.8% viable and 28.8% dead, compared with 2D, 70% viable and 12.3% dead), which was Gal inhibitable (2F, 64.0% viable and 13.4% dead) but reduced in magnitude compared with that of the parental strain (2B) (confirmed in three separate studies). The ldlD.Lec1 mutant (2G, 81.8% viable and 3.2% dead) was resistant to amebic cytolytic activity (2H, 62.2% viable and 8.3% dead), and viability was unaffected by Gal (2I, 63.5% viable and 8.2% dead). Amebic cytolysis of Lec2 mutants (2K, 32.3% viable and 32.8% dead, compared with 2J, 71.1% viable and 6.1% dead) was comparable to that of parental target cells (2B). However, as with the adherence studies, amebic cytolysis of Lec2 mutants was less inhibited by an equal concentration of Gal (2L, 53.1% viable and 17.1% dead) compared with the parental strain (2C).

Amebic cytolysis of target CHO cells was also determined by direct hemacytometer counts (Table 3). Trophozoites demonstrated Gal-inhibitable killing of parental Gat$^-$2 cells, Lec1 mutants, and Lec2 mutants. By this method, ldlD.Lec1 mutants were again found to be essentially resistant to the cytolytic activity of axenic *E. histolytica* trophozoites. Amebic killing of the Lec2 mutant targets required higher concentrations of Gal for complete inhibition (viability of target Lec2 mutants increased from 47.8% at 10 mg of Gal per ml to 62.9, 71.2, and 86.2% at 20, 30, and 40 mg of Gal per ml, respectively; $P < 0.01$ for $\geq$20 mg/ml compared with control medium [Table 3]).

DISCUSSION

By studying adherence and cytolytic activities of viable *E. histolytica* trophozoites and binding of purified amebic Gal-GalNAc lectin to a panel of different CHO glycosylation mutants, we have further defined the binding specificity of the lectin and provided additional evidence of its direct association with *E. histolytica* cytolytic activity. It seems clear that lactosamine units of N-linked carbohydrates are the major receptors for amebic binding at 4°C. This was also concluded by Li et al. (13) in their study of amebic interactions with three CHO glycosylation mutants carrying the same glycosylation defects as the Lec1, Lec2, and Lec8 mutants in our panel (35). By comparing amebic and lectin binding as well as cytolysis of glycosylation mutants having additional carbohydrate alterations, the present study has revealed that the amebic Gal-GalNAc adherence lectin prefers branched N-linked carbohydrates (reduced in the Lec4 mutant [38]) lacking terminal sialic acid, as in Lec2 and Lec3 mutants. In addition, although the presence of α-1,3 fucose residues on nonsialylated lactosamines (LEC12) reduces amebic adherence, the presence of α-2,3-sialylated, α-1,3-fucosylated lactosamine as in LEC11 was a slightly preferable confirmation for amebic adherence.

Perhaps more importantly, this study has shown that at 37°C, under conditions more closely approximating physiological conditions, the lack of N-linked lactosamine units barely affects amebic binding to Lec1 cells (Table 2) and that efficient cytolysis of Lec1 cells ensues (Table 3, Fig. 1). The properties of the ldlD.Lec1 mutant provide strong evidence that the susceptibility of Lec1 cells to *E. histolytica* at 37°C is mediated by O-linked carbohydrates. It is unlikely that an alternative *E. histolytica* adhesin mediated cytolysis of Lec1 CHO cell mutants, as the cytolytic activity observed was entirely Gal inhibitable. In contrast to Lec1 cells, the ldlD.Lec1 mutant is virtually resistant to *E. histolytica* adherence and cytolysis at 37°C. The N-linked carbohydrates of Lec1 and ldlD.Lec1 cells should be identical, because with respect to N-linked carbohydrates, the Lec1 mutation is epistatic to the *ldlD* mutation (36). Thus the mutants differ solely in that Lec1 cells should synthesize a normal spectrum of O-linked carbohydrates (32), whereas ldlD.Lec1 cells in serum-free medium should not synthesize O-linked carbohydrates at all (12; Krieger et al., in press). A small amount of O-linked carbohydrate synthesis may account for the barely detectable binding and cytolysis observed with the ldlD.Lec1 mutant.

When terminal sialic acid residues were removed by neuraminidase treatment of parental CHO cells, amebic adherence mimicked that observed with the Lec2 or Lec3 CHO cell mutants. In addition to confirming the importance of Gal residues in lectin binding, these studies suggest that the *E. histolytica* neuraminidase enzyme could have an accessory function in adherence by modifying target cell receptors to optimize Gal-GalNAc lectin binding. Udezulu and Leitch (40) demonstrated that *E. histolytica* trophozoites liberated sialic acid from *N*-acetylneuraminlactose or commercial mucins and that parasite neuraminidase activity was highly surface plasma membrane associated. Another protozoan, *Trypanosoma cruzi*, contains a neuraminidase that removes sialic acid residues from target myocardial and vascular endothelial cells (15).

We recently isolated the Gal-GalNAc lectin of *E. histolytica* and demonstrated its ability to competitively inhibit the binding of viable trophozoites to target CHO cells (18). This indicated that the adherence lectin in soluble or membrane-bound states binds to similar CHO cell surface carbohydrate receptors. Binding of purified soluble lectin to target CHO cells at 4°C, measured with an iodinated anti-lectin monoclonal antibody, correlated exactly with cell adherence in the four CHO cell lines studied. Gal-inhibitable lectin binding was reduced for the Lec1 mutant, essentially absent for the ldlD.Lec1 mutant, and increased for the Lec2 mutant. These binding studies cannot be performed at 37°C due to endocytosis of bound lectin by CHO cells. This study confirms previous reports (18, 21, 24) that the Gal-GalNAc lectin (16b, 18) exclusively mediates amebic adherence in the CHO cell model. The relevance of this amebic adherence lectin to human disease is supported by its antigenicity; the 170-kilodalton heavy subunit of the lectin is the most prominantly recognized protein upon immunoblotting and immunoprecipitation with large numbers of immune sera obtained

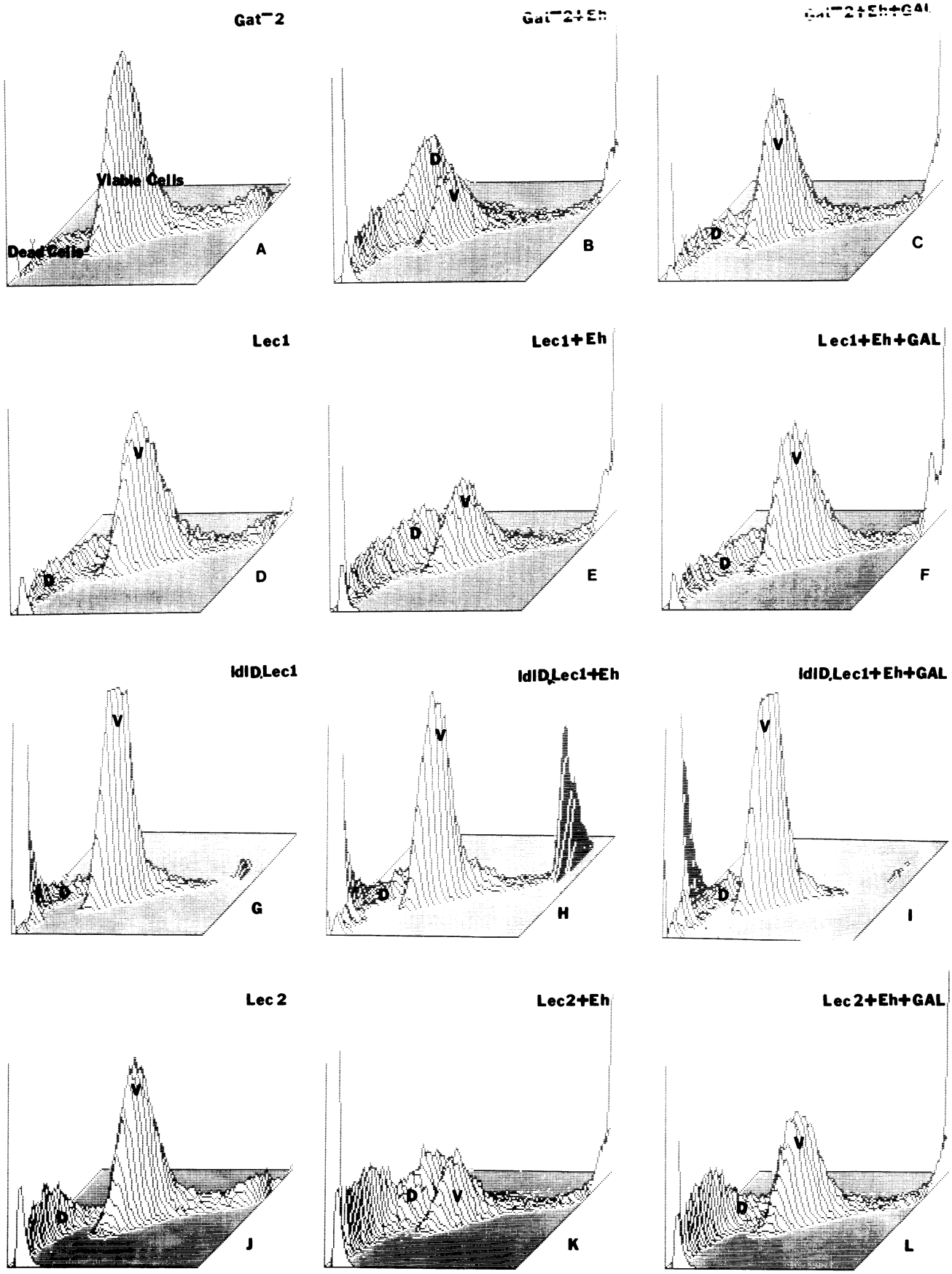


FIG. 2. Gal-inhibitable cytolysis of target CHO cells by *E. histolytica* trophozoites (Eh) as determined by cell sorting. The vertical *z* axis is cell number, and the *x* and *y* axes are size and density, respectively. The peaks seen in the far right in panels C, E, F, H, K, and L are *E. histolytica* trophozoites. (A, D, G, and J) CHO cells incubated without amebae; (B, E, H, and K) CHO cells with amebae; (C, F, I, and L) amebae and CHO cells with Gal.

from patients cured of invasive amebiasis from diverse geographic areas (10, 16a, 17).

Previously, we demonstrated that *E. histolytica* trophozoites deliver a contact-dependent lethal hit to target CHO cells by use of trypan blue exclusion criteria, release of cytoplasmic [^{111}In]indium oxine from target CHO cells, and CHO cell Fura 2 fluorescence (21, 23, 24). The relevance of the CHO cell cytolysis model is supported by the ability of Gal or GalNAc to inhibit amebic cytolysis of human neutrophils, macrophages, lymphocytes, and cultivated Chang liver cells (24, 29, 30). By using Gal to elute adherent target cells from amebae and the cell sorter, we now report a new method to directly measure the population of target cells which receive a lethal hit by trophozoites but have not yet been ingested. Results with previous methodologies (Table 3) show the validity of the new method, which is clearly superior to previous methods in speed of data acquisition and presentation and should be generally useful for determining cytotoxicity.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-18841 (to Jonathan I. Ravdin) from the National Institute for Allergy and Infectious Diseases, by Public Health Service grant NCI-30645 (to Pamela Stanley) from the National Cancer Institute, and by a grant from the Lucille P. Markey Charitable Trust (to William A. Petri, Jr.). William A. Petri, Jr., is a Lucille P. Markey Fellow.

We acknowledge Barbara Taylor and Susan Roscoe for expert secretarial assistance.

ADDENDUM IN PROOF

While this manuscript was under review, E. Li, A. Becker, and S. L. Stanley, Jr. (Infect. Immun. **57**:8-12, 1989) reported that a CHO cell mutant deficient in N-linked acetyllactosamine units (RIC^R 15B, analogous to the Lec1 in our studies) was partially resistant to killing by *E. histolytica* trophozoites (strain HM1:IMSS). The RIC^R 15B CHO mutants could be lysed at high ameba/CHO cell ratios, which was interpreted by the researchers as evidence against carbohydrate-specific adherence being an absolute requirement for cytolysis. However, that finding was consistent with our demonstration that O- as well as N-linked surface carbohydrates can serve as receptors for amebic Gal-GalNAc lectin-mediated cytolytic activity.

LITERATURE CITED

1. **Campbell, C., and P. Stanley.** 1984. A dominant mutation to ricin-resistance in CHO cells induces UDP-GlcNAc glycopeptide *N*-acetylglucosaminyltransferase III activity. J. Biol. Chem. **261**:13370–13378.
2. **Chadee, K., and E. Meerovitch.** 1984. The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica*. Am. J. Trop. Med. Hyg. **33**:47–54.
3. **Chadee, K., W. A. Petri, D. J. Innes, and J. I. Ravdin.** 1987. Rat and human colonic mucins bind to and inhibit the adherence lectin of *Entamoeba histolytica*. J. Clin. Invest. **80**:1245–1254.
4. **Chadee, K., W. A. Petri, M. Johnson, E. Orozco, and J. I. Ravdin.** 1988. Binding and internalization of purified rat colonic mucins by the Gal/GalNAc adherence lectin of *Entamoeba histolytica*. J. Infect. Dis. **158**:398–406.
5. **Deutscher, S. L., and C. B. Hirschberg.** 1986. Mechanism of galactosylation in the Golgi apparatus of Chinese hamster ovary cells mutant in translocation of UDP-galactose across Golgi vesicle membranes. J. Biol. Chem. **261**:96–100.
6. **Deutscher, S. L., N. Nuwayhid, P. Stanley, E. B. Briles, and C. B. Hirschberg.** 1984. Translocation across Golgi membranes. A CHO glycosylation mutant deficient in CMP-sialic acid transport. Cell **39**:295–299.
7. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. **72**:431–432.
8. **Green, E. D., R. M. Bradbeck, and J. U. Baenziger.** 1987. Lectin affinity high-performance liquid chromatography. Interactions of *N*-glycanase-released oligosaccharides with *Ricinus communis* agglutinin I and *Ricinus communis* agglutinin II. J. Biol. Chem. **262**:12030–12039.
9. **Howard, D. R., M. Fukuda, M. N. Fukuda, and P. Stanley.** 1987. The GDP-fucose: *N*-acetylglucosamine 3-α-L-fucosyltransferases of LEC11 and LEC12 Chinese hamster ovary mutants exhibit novel specificities for glycolipid substrates. J. Biol. Chem. **262**:16830–16837.
10. **Joyce, M. P., and J. I. Ravdin.** 1988. Antigens of *Entamoeba histolytica* recognized by immune sera from liver abscess patients. Am. J. Trop. Med. Hyg. **38**:74–80.
11. **Joyce, M. P., and J. I. Ravdin.** 1988. Pathology of human amebiasis, p. 129–146. *In* J. I. Ravdin (ed.), Amebiasis: human infection by *Entamoeba histolytica*. John Wiley & Sons, Inc., New York.
12. **Kingsley, D. M., K. F. Kozarsky, L. Hobbie, and M. Krieger.** 1986. Reversible defects in O-linked glycosylation and ldl receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase mutant. Cell **44**:749–759.
13. **Li, E., A. Becker, and S. L. Stanley.** 1988. Use of Chinese hamster ovary cells with altered glycosylation patterns to define the carbohydrate specificity of *Entamoeba histolytica* adhesion. J. Exp. Med. **167**:1725–1730.
14. **Li, E., R. Gibson, and S. Kornfeld.** 1980. Structure of an unusual complex-type oligosaccharide isolated from Chinese hamster ovary cells. Arch. Biochem. Biophys. **199**:393–399.
15. **Libby, P., J. Alroy, and M. E. A. Pereira.** 1986. A neuraminidase from *Trypanosoma cruzi* removes sialic acid from the surface of mammalian myocardial and endothelial cells. J. Clin. Invest. **77**:127–135.
16. **Murty, J. L. N., F. J. Downs, and W. Pigman.** 1978. Rat-colonic mucus glycoprotein. Carbohydr. Res. **61**:139–145.
16a. **Petri, W. A., Jr., J. Broman, G. Healy, T. C. Quinn, and J. I. Ravdin.** 1989. Antigenic stability and immunodominance of the Gal/GalNAc adherence lectin of *Entamoeba histolytica*. Am. J. Med. Sci. **297**:163–165.
16b. **Petri, W. A., Jr., M. D. Chapman, T. Snodgrass, B. J. Mann, J. Broman, and J. I. Ravdin.** 1989. Subunit structure of the galactose and N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. J. Biol. Chem. **264**:3007–3012.
17. **Petri, W. A., M. P. Joyce, J. Broman, R. D. Smith, C. F. Murphy, and J. I. Ravdin.** 1987. Recognition of the galactose or *N*-acetylgalactosamine binding lectin of *Entamoeba histolytica* by human immune sera. Infect. Immun. **55**:2327–2331.
18. **Petri, W. A., R. D. Smith, P. H. Schlesinger, and J. I. Ravdin.** 1987. Isolation of the galactose-binding lectin which mediates the *in vitro* adherence of *Entamoeba histolytica*. J. Clin. Invest. **80**:1238–1244.
19. **Ravdin, J. I.** 1986. Pathogenesis of disease with *Entamoeba histolytica*. Studies of adherence toxins and contact-dependent cytolysis. Rev. Infect. Dis. **8**:247–260.
20. **Ravdin, J. I., B. Y. Croft, and R. L. Guerrant.** 1980. Cytopathogenic mechanisms of *Entamoeba histolytica*. J. Exp. Med. **152**:377–390.
21. **Ravdin, J. I., and R. L. Guerrant.** 1981. The role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes. J. Clin. Invest. **68**:1305–1313.
22. **Ravdin, J. I., J. E. John, L. I. Johnston, D. J. Innes, and R. L. Guerrant.** 1985. Adherence of *Entamoeba histolytica* trophozoites to rat and human colonic mucosa. Infect. Immun. **48**: 292–297.
23. **Ravdin, J. I., F. Moreau, J. A. Sullivan, W. A. Petri, and G. L. Mandell.** 1988. The relationship of free intracellular calcium ions to the cytolytic activity of *Entamoeba histolytica*. Infect. Immun. **56**:1505–1512.
24. **Ravdin, J. I., C. F. Murphy, R. A. Salata, R. L. Guerrant, and**

E. L. Hewlett. 1985. *N*-Acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. I. Partial purification and relation to amoebic virulence in vitro. J. Infect. Dis. **151**:804–815.

25. **Ravdin, J. I., W. A. Petri, C. F. Murphy, and D. R. Smith.** 1986. Production of mouse monoclonal antibodies which inhibit in vitro adherence of *Entamoeba histolytica* trophozoites. Infect. Immun. **53**:1–5.
26. **Ripka, J., S.-I. Shin, and P. Stanley.** 1986. Decreased tumorigenicity correlates with expression of altered carbohydrates in Lec9 CHO cells. Mol. Cell. Biol. **6**:1268–1275.
27. **Robertson, M. A., J. R. Etchison, J. S. Robertson, D. F. Summers, and P. Stanley.** 1978. Specific changes in the oligosaccharide moieties of VSV grown in different lectin-resistant CHO cells. Cell **13**:515–526.
28. **Rosenwald, A. G., P. Stanley, and S. S. Krag.** 1989. Control of carbohydrate processing: increased β-1,6 branching in N-linked carbohydrates of Lec9 CHO mutants appears to arise from a defect in oligosaccharide-dolichol biosynethsis. Mol. Cell. Biol. **9**:914–924.
29. **Salata, R. A., R. D. Pearson, and J. I. Ravdin.** 1985. Interaction of human leukocytes with *Entamoeba histolytica*: killing of virulent amebae by the activated macrophage. J. Clin. Invest. **76**:491–499.
30. **Salata, R. A., and J. I. Ravdin.** 1986. The interaction of human neutrophils and *Entamoeba histolytica* increases cytopathogenicity for liver cell monolayers. J. Infect. Dis. **154**:19–26.
31. **Slomiany, B. L., V. L. N. Murty, and A. Slomiany.** 1980. Isolation and characterization of oligosaccharides from rat colonic mucous glycoproteins. J. Biol. Chem. **225**:9719–9723.
32. **Stanley, P.** 1980. Altered glycolipids of CHO cells resistant to wheat germ agglutinin. Am. Chem. Soc. Symp. Ser. **128**: 213–221.
33. **Stanley, P.** 1983. Lectin-resistant CHO cells: selection of new mutant phenotypes. Somatic Cell Genet. **9**:593–608.
34. **Stanley, P.** 1984. Glycosylation mutants of animal cells. Annu. Rev. Genet. **18**:525–552.
35. **Stanley, P.** 1985. Membrane mutants of animal cells: rapid identification of those with a primary defect in glycosylation. Mol. Cell. Biol. **5**:923–929.
36. **Stanley, P.** 1989. Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. Mol. Cell. Biol. **9**: 377–383.
37. **Stanley, P., T. Sudo, and J. P. Carver.** 1980. Differential involvement of cell surface sialic acid residues in wheat germ agglutinin binding to parental and wheat germ agglutinin-resistant Chinese hamster ovary cells. J. Cell Biol. **85**:60–69.
38. **Stanley, P., G. Vivona, and P. H. Atkinson.** 1984. ^{1}H-NMR spectroscopy of carbohydrates from the G glycoprotein of VSV grown in parental and Lec4 CHO cells. Arch. Biochem. Biophys. **230**:363–374.
39. **Tsutsumi, V., R. Mena-Lopez, F. Anaya-Velazquez, and A. Martinez-Palomo.** 1984. Cellular basis of experimental amebic liver abscess formation. Am. J. Pathol. **117**:81–91.
40. **Udezulu, I. A., and G. J. Leitch.** 1987. A membrane-associated neuraminidase in *Entamoeba histolytica* trophozoites. Infect. Immun. **55**:181–186.
41. **Walsh, J. A.** 1986. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. Rev. Infect. Dis. **8**:228–238.