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Chinese Hamster Ovary Cells Deficient in *N*-Acetylglucosaminyltransferase I Activity Are Resistant to *Entamoeba histolytica*-Mediated Cytotoxicity

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To study the relationship between carbohydrate-specific amebic cytoadherence and ameba-mediated cytotoxicity, we measured *Entamoeba histolytica* trophozoite-mediated cytolysis directed against a panel of four Chinese hamster ovary (CHO) cell lines that have defined alterations in their glycosylation patterns. We recently measured amebic trophozoite adherence to this panel of CHO cells and showed that trophozoites bind variant cells (RIC^R 15B), which are deficient in Asn-linked *N*-acetyllactosamine units, at 12% of the level observed for wild-type cells (E. Li, A. Becker, and S. L. Stanley, J. Exp. Med 167:1725–1730, 1988). Using a ^{51}Cr release assay to measure trophozoite-mediated cytolysis, we demonstrate in this study that RIC^R 15B cells are less susceptible to trophozoite-mediated cytolysis than are wild-type cells. In addition, we found that *N*-acetyllactosamine, which inhibits trophozoite adherence to CHO cells, also inhibited trophozoite-mediated cytolysis of wild-type cells. These studies indicate that surface carbohydrates on target cells can influence susceptibility to ameba-mediated cytotoxicity. This panel of CHO cells provides a useful model system for investigating the role of glycoconjugates in mediating amebic interactions with mammalian cells.

Invasive amebiasis is characterized by disruption and invasion of the colonic mucosa by *Entamoeba histolytica* trophozoites (8, 16). In hepatic amebic abscesses, extensive tissue necrosis is often found with few or no polymorphonuclear leukocytes, which suggests that cytopathogenic properties of the trophozoites are important in producing disease (16). Amebic trophozoites are lethal to a number of cultured mammalian cell lines (5, 15). This property correlates well with in vivo measurements of strain virulence (12). For this reason, the mechanisms by which amebic trophozoites exert their cytopathic effects have been the subject of much study. Microscopic analysis of ameba-destroying monolayer cells has shown that target cells in direct contact with trophozoites exhibit extensive blebbing, loss of normal shape, and uptake of trypan blue, whereas neighboring target cells not in direct contact remain intact. These findings suggest that damage of target cells is initiated by a contact-dependent process (17) and that the ameba cytolethal effect usually precedes ameba phagocytosis (17).

On the basis of evidence that trophozoite-mediated cytolysis is initiated by contact between trophozoites and target cells, it seems reasonable to propose that mechanisms facilitating adherence of trophozoites to target cells would also facilitate cytolysis. We previously compared adherence of amebic trophozoites to a panel of variant CHO cell lines that have defined alterations in their glycosylation patterns (9). We demonstrated that monolayers of WGA^R 1021 cells, which are deficient in membrane sialic acid and have increased galactose at the nonreducing termini (2), bound twice as many trophozoites as did monolayers of wild-type cells. WGA^R 13 cells, which are deficient in membrane-bound sialic acid and galactose and have increased N-acetylglucosamine residues at the nonreducing termini (2), bound 30% as many trophozoites as were bound by wild-type cells. RIC^R 15B cells, which lack Asn-linked complex oligosaccharides and accumulate oligomannosyl units in their glycoproteins because of a deficiency in *N*-acetylglucosaminyltransferase I (6, 7, 10), bound trophozoites least well (12% of wild-type levels). Furthermore, *N*-acetyllactosamine and lactose inhibited adherence at concentrations at which sialyllactose, melibiose, and chitobiose did not. These studies suggest that terminal *N*-acetyllactosamine units on Asn-linked complex-type chains provide the major receptors for amebic adhesion to CHO cells (9). To examine the relationship between this mechanism of adherence and ameba-mediated cytolysis, we used this panel of CHO cell lines as target cells in a ^{51}Cr release assay.

MATERIALS AND METHODS

Cells. *E. histolytica* HM1-IMSS trophozoites were kindly provided by Jonathan Ravdin. *E. histolytica* HK-9 and the *E. histolytica*-like Laredo strain were obtained from the American Type Culture Collection (Rockville, Md.). The ameba were grown in TYI-S-33 medium as described previously (3). The wild-type and variant CHO cells were provided by Stuart Kornfeld and grown as described previously (2). Before amebic lysis assays, the CHO cells were removed by light trypsinization and placed in spinner cultures at 1×10^5 to 2×10^5 cells per ml for 48 h.

Ameba-mediated cytotoxicity assays. Target (CHO) cells from 48-h spinner cultures were centrifuged and suspended in α-minimal essential medium with 10% fetal calf serum at a concentration of 10^7 cells per ml and were labeled with 100 μCi of ^{51}Cr (sodium chromate, 350 to 600 mCi of chromium per mg; Amersham Corp., Arlington Heights, Ill.) per ml for 90 min at 37°C with agitation every 15 min. The cells were washed five times in cold α-minimal essential medium with 5.7 mM cysteine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 1% bovine serum albumin (test medium) and adjusted to 4×10^5/ml in test medium. Cell viability was >90% as determined by trypan blue exclusion. Amebic trophozoites (72-h cultures) were har-

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vested by chilling and centrifugation. The trophozoites were washed twice with test medium and suspended at concentrations ranging from 2×10^5 to 8×10^5 cells per ml in test medium. Cell viability was >90% as determined by trypan blue exclusion. The assay was performed in quadruplicate in flat-bottomed 96-well plates by combining 100 μl of target cells with 100 μl of trophozoites. After incubation at 37°C in 5% CO_2 for 2 to 6 h, 100 μl of supernatant was removed and counted in a gamma counter (Auto-Gamma spectrometer; Packard Instrument Co., Inc., Rockville, Md.). Controls included target cells incubated with 100 μl of test medium to measure spontaneous release of counts and target cells incubated with 100 μl of 2% Triton X-100 to measure maximal release of counts. The percent ameba-mediated cytotoxicity was calculated as follows: [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100.

Spontaneous release ranged from 8 to 12% of the total radioactivity. In a given experiment, the standard deviation of quadruplicate values did not exceed 10%. The relative susceptibility of the variant CHO cells compared with that of wild-type cells was expressed as a relative killing index, which was calculated for a given experiment as follows: ameba-mediated cytotoxicity against mutant cells/ameba-mediated cytotoxicity against wild-type cells.

Inhibition of cytotoxicity with sugars. Saccharides were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Pfahnstiehl Laboratories (Waukegan, Ill.). Ameba were suspended at 4×10^5 cells per ml in test medium containing inhibitors at 100 mM for 5 min before addition to the microdilution plate. The final pHs of all solutions, e.g., sialyllactose, were adjusted to 7.4. A 100-μl amount of the ameba suspension was added to 100 μl of wild-type CHO target cells to give a final concentration of 50 mM inhibitor, and ameba-mediated cytotoxicity was measured as described above. Control assays using ameba suspended in test medium alone were conducted simultaneously. The spontaneous release of counts by the target cells was not affected by incubation in the presence of any of the saccharide inhibitors tested.

Statistical analysis. Differences between the means of experimental groups were determined by a two-tailed Student *t* test. Differences were considered significant at the level of $P < 0.05$.

Phase-contrast photomicrographs. Cells were photographed on Polaroid type 107 film through a Nikon phase-contrast microscope.

RESULTS

Amebic trophozoite-mediated cytolysis of CHO lines. We analyzed amebic trophozoite-mediated cytotoxicity by measuring specific release of ^{51}Cr from radiolabeled CHO cells in an assay similar to one described in which Chang liver cells were used as target cells (13) and similar to assays used to study lymphocyte-mediated cytolysis (1, 11). These assays were conducted in the absence of serum in order to avoid interference by serum glycoproteins. Trophozoites appeared to remain viable after 6 h of incubation as determined by trypan blue exclusion. When ^{51}Cr-labeled wild-type CHO cells were used as target cells, the degree of trophozoite-mediated cytolysis exhibited by two strains of *E. histolytica*, HM1-IMSS and HK-9, and by the nonpathogenic *E. histolytica*-like Laredo strain (Fig. 1) appeared to correlate with in vivo measurements of strain virulence (12). With 4×10^4 trophozoites per well (effector/target ratio of 1:2), trophozoite-mediated cytolysis after 2 h was 37% for HM1-IMSS, 11% for HK-9, and 0.5% for Laredo. After 4 h, trophozoite-mediated cytolysis was 53% for HM1-IMSS, 14% for HK-9, and 0.6% for Laredo.

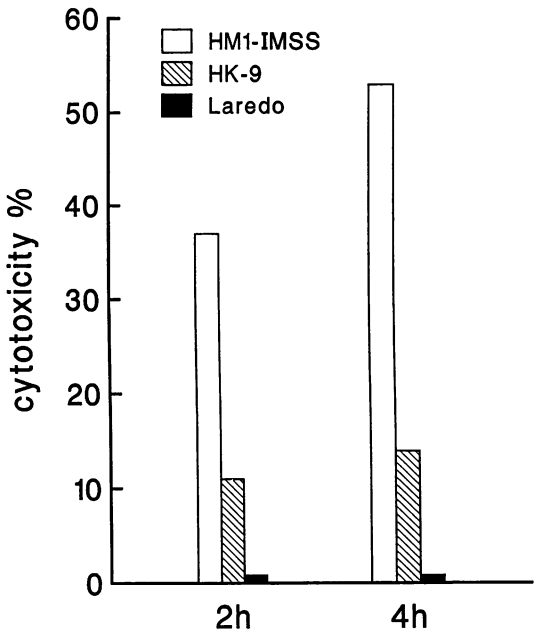

FIG. 1. Trophozoite-mediated cytolysis directed against wild-type CHO cells by the HM1-IMSS, HK-9, and nonpathogenic Laredo strains. Trophozoite-mediated cytotoxicity was measured as described in Materials and Methods, using 4×10^4 trophozoites per well (effector/target ratio of 2:1).

Since amebic trophozoites adhered most poorly to RIC^R 15B cells (9), we compared the susceptibility of RIC^R 15B cells to trophozoite-mediated cytolysis with that of wild-type cells. We used HM1-IMSS trophozoites because this strain of trophozoites demonstrated significant cytolysis against wild-type cells. Significant differences in the susceptibilities of RIC^R 15B and wild-type cells to trophozoite-mediated cytolysis were observed at a concentration of 2×10^4 trophozoites per well (effector/target ratio of 1:1) after 3 h of incubation (8 ± 4% versus 19 ± 7%, $P < 0.05$) and after 6 h of incubation (17 ± 7% versus 39 ± 10%, $P < 0.005$) (Fig. 2). Significant differences were also observed at a concentration of 4×10^4 trophozoites per well (effector/target ratio of 2:1) after 3 h of incubation (28 ± 11% versus 44 ± 10%, $P < 0.005$). After 6 h of incubation with 4×10^4 trophozoites per well, the difference in ^{51}Cr release between the two cell lines (58 ± 9% and 69 ± 8%) was not significant. At lower concentrations of trophozoites, the level of cytolysis observed for wild-type CHO cells (5% at 10^4 trophozoites per well) was too low to make comparative studies feasible.

On microscopic inspection of the wells, we observed a clear difference in the appearance of trophozoites incubated with wild-type CHO cells compared with trophozoites incubated with RIC^R 15B cells. After 30 min of incubation of HM1-IMSS trophozoites with wild-type cells at 37°C, the trophozoites began to aggregate (Fig. 3A). In contrast, the trophozoites remained dispersed even after several hours of incubation with RIC^R 15B cells (Fig. 3B).

Using conditions in which RIC^R 15B cells could be shown to be less susceptible than wild-type cells to trophozoite-mediated cytolysis, we examined the relative susceptibilities of WGA^R 1021 cells (deficient in membrane-bound sialic

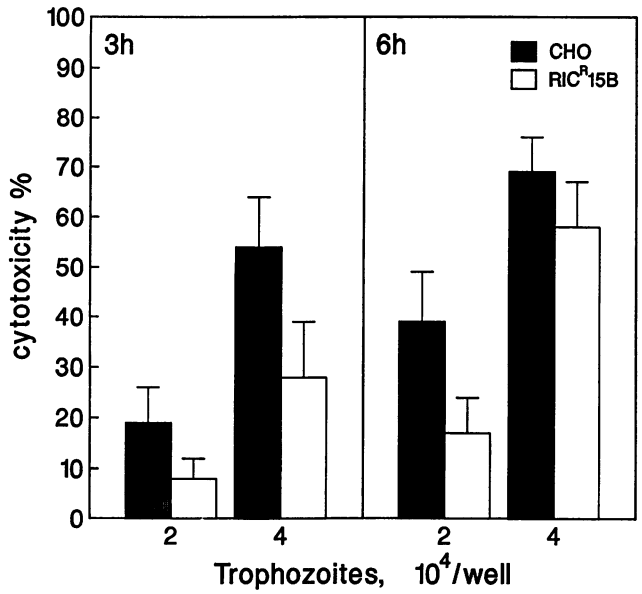

FIG. 2. Comparison of HM1-IMSS-mediated cytotoxicity directed against wild-type and RIC^R 15B cells. Trophozoite-mediated cytotoxicity was measured as described in Materials and Methods. Each point represents the mean of five experiments.

acid) and of WGA^R 13 cells (deficient in both membrane-bound sialic acid and galactose). For comparison of experiments performed on separate days, the results are expressed relative to the trophozoite-mediated cytolysis observed for wild-type CHO cells in a given experiment (killing index). The relative susceptibilities of WGA^R 1021, WGA^R 13, and RIC^R 15B cells after incubation for 6 h with 2×10^4 trophozoites per well were 0.61 ± 0.25 ($P < 0.01$), 0.84 ± 0.27 (not significant), and 0.45 ± 0.16 ($P < 0.005$) (Fig. 4).

Effect of various sugars on amebic cytotoxicity. Various sugars were tested for capacity to affect the cytotoxic activity of amebic trophozoites on ^{51}Cr-labeled wild-type CHO cells (Fig. 5). Of the saccharides examined, both 50 mM *N*-acetyllactosamine (Gal-1 → 4GlcNAc, 23 ± 21% cytotoxicity, $P < 0.025$) and 50 mM lactose (Gal-1 → 4Glu, 33 ± 23% cytotoxicity, $P < 0.05$%) showed a consistent

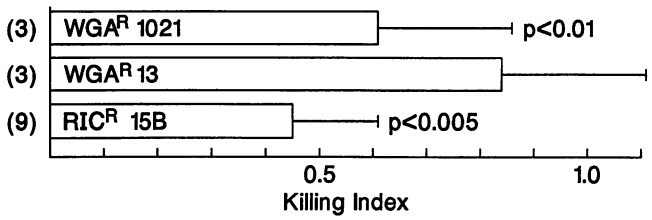

FIG. 4. Relative susceptibilities of variant CHO cell lines WGA^R 1021, WGA^R 13, and RIC^R 15B to HM1-IMSS-mediated cytolysis. Results are normalized with respect to wild-type cells for a given experiment and expressed as a killing index. Trophozoites (2×10^4) were incubated with CHO target cells (effector/target ratio of 1:1) for 6 h as described in Materials and Methods.

inhibitory effect compared with results of control assays (61 ± 8% cytotoxicity). We showed previously that at a concentration of 50 mM, these two saccharides were the most effective saccharide inhibitors of amebic adhesion (9). In one series of experiments, serial dilutions of the sugars showed that significant inhibition was observed only at a concentration of 50 mM. Melibiose at 50 mM (Gal-α1 → 6Glu, 40 ± 32% cytotoxicity) did not demonstrate reproducible inhibition. Neither 50 mM chitobiose (GlcNAc-1 → 4GlcNAc, 50 ± 17% cytotoxicity) nor 50 mM sialyllactose (NANA-α2 → 3Gal-1 → 4Glu, 53 ± 11% cytotoxicity) had an inhibitory effect. (Abbreviations: Gal, galactose; glu, glucose; GlcNAc, *N*-acetylglucosamine; NANA, *N*-acetylneuraminic acid.)

DISCUSSION

It has been proposed that the interaction of ameba with eucaryotic target cells follows three basic steps: adherence, followed by cytolysis, followed by phagocytosis (17). Furthermore, amebic lectins may play an important role in mediating adherence (14). To test the hypothesis that carbohydrate-specific adherence plays an important role in ameba-mediated cytolysis, we examined the relative susceptibility to ameba-mediated cytolysis among a panel of variant CHO cells with defined alterations in glycosylation patterns. We have previously characterized this panel of cells with respect to trophozoite adherence and have shown that adherence, when measured at 4°C, correlates with the presence of terminal *N*-acetyllactosamine units on CHO cells.

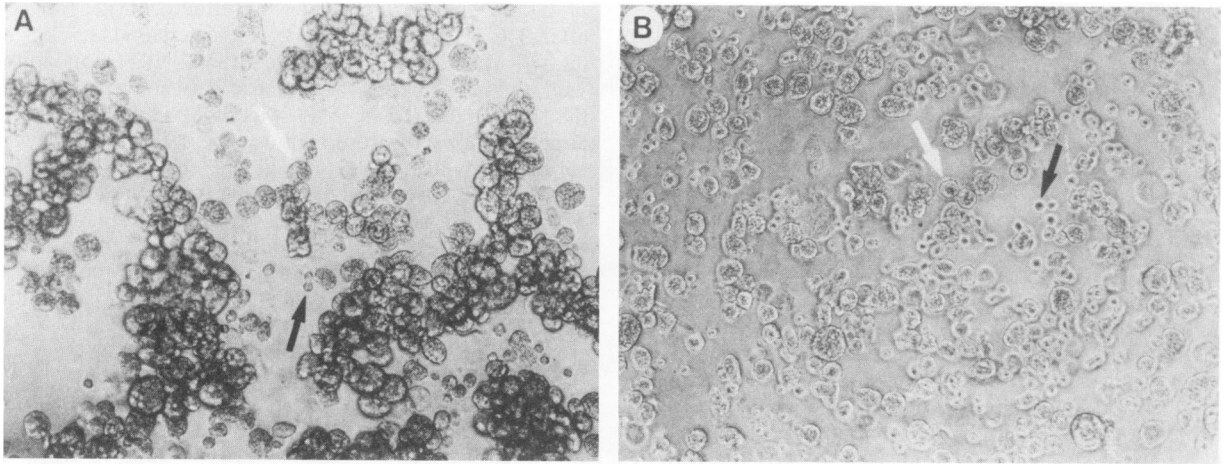

FIG. 3. Phase-contrast photomicrographs of HM1-IMSS trophozoites incubated for 30 min with wild-type CHO cells (A) and RIC^R 15B cells (B). White arrows point to trophozoites; black arrows point to CHO target cells. Magnification, ×85.

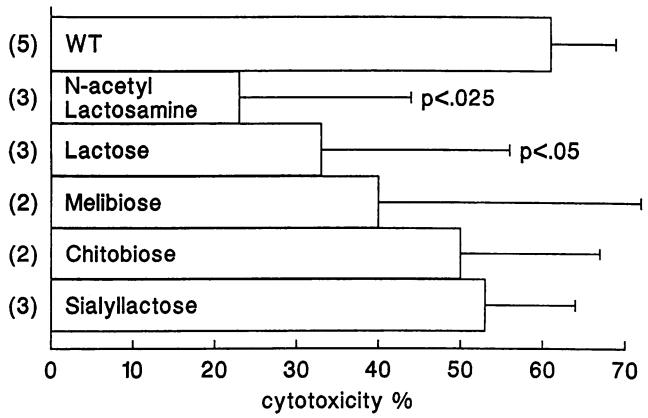

FIG. 5. Effect of saccharides (50 mM) on ameba-mediated cytotoxicity directed against wild-type CHO cells. Bars represent the mean ± standard deviation for the number of experiments shown in parentheses on the vertical scale. A total of 2×10^4 trophozoites/per well were incubated for 6 h at 37°C with wild-type CHO cells.

The lysis of cells by ameba has been studied in vitro by cell culture monolayer experiments in which destruction of monolayers is monitored and by cell suspension experiments in which release of chromium-51 or indium-111 oxide from radiolabeled cells is measured (15). We used the latter method because release of viable cells from the monolayer by proteolysis complicates interpretation of the first method (15). Our assays differed from those previously described in that they were conducted in the absence of serum to avoid interference from carbohydrates in serum glycoproteins (12, 17, 18).

We found that RIC^R 15B cells were more resistant than wild-type cells to trophozoite-mediated cytolysis. RIC^R 15B cells have a selective deficiency in Asn-linked *N*-acetyllactosamine units and bound trophozoites least well of the variant cells studied. Although one cannot exclude selective toxic effects of saccharide inhibitors, the observation that *N*-acetyllactosamine and lactose inhibited cytolysis at concentrations at which no significant inhibition was observed for sialyllactose and chitobiose further supports the hypothesis that carbohydrate-specific adherence plays a role in trophozoite-mediated cytolysis. The observation that RIC^R 15B cells were lysed when the trophozoite concentration was increased argues against carbohydrate-specific adherence being an absolute requirement for cytolysis. Although adherence specific for *N*-acetyllactosamine units appears to be the major mechanism by which trophozoites adhere to CHO cells at 4°C, other mechanisms of adherence may also be important at 37°C.

Although WGA^R 13 cells also bound trophozoites less well than did wild-type cells, a significant difference in susceptibility to cytolysis could not be reproducibly demonstrated. One explanation is that the difference in adherence (30% of wild-type levels) is not sufficient for a difference in cytolysis to be detected with this assay. Although WGA^R 1021 cells bound twice as many trophozoites as did wild-type cells, these cells appeared to be somewhat less susceptible to cytolysis. It is noteworthy that Feingold et al. (4) have reported partial purification of a sialic acid-sensitive toxic factor which appears to correlate with virulence. Thus, carbohydrate determinants may mediate mechanisms involved in cytolysis other than adherence.

Our studies demonstrate a clear difference in how *E. histolytica* trophozoites interact with two cell lines expressing different surface glycoconjugates. They show that surface glycoconjugates can influence susceptibility to ameba-mediated cytolysis and that the differences in susceptibility can be correlated with the phenomenon of *N*-acetyllactosamine-specific adherence. This panel of variant cells should provide a useful model system for further investigation into the role of glycoconjugates in mediating amebic interactions with mammalian cells.

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