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In Vitro Model of Attachment of *Giardia intestinalis* Trophozoites to IEC-6 Cells, an Intestinal Cell Line

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Attachment of giardias to intestinal cells has been difficult to study because of a lack of a convenient in vitro model. We developed an assay for attachment of radiolabeled trophozoites to IEC-6 cells that can be done in microtiter trays. Attachment was confirmed by scanning and transmission electron microscopy. Trophozoites remained attached to the IEC-6 cells for 24 h with little evidence of damage to the IEC-6 cells. Preincubation of trophozoites with cytochalasins A, B, and D reduced attachment to approximately 20% of that of controls, whereas colchicine had no effect. Chelation of divalent cations with EDTA and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] reduced attachment to 24 and 26% of control values, respectively, and incubation at 4°C reduced attachment to 7% of the value for controls incubated at 37°C. Glutaraldehyde fixation of trophozoites or IEC-6 cells resulted in significantly diminished attachment to the live substrate (17 and 40% of control values, respectively). Coincubation of IEC-6 cells and trophozoites on a rotary shaker resulted in detachment of 40% of trophozoites, but EDTA, EGTA, glutaraldehyde fixation of trophozoites, and low temperature diminished attachment markedly and significantly. Similar results were obtained in selected experiments with three strains of giardia.

Attachment of giardia trophozoites to the luminal side of the small-intestinal epithelial cell membrane is a cardinal feature of infection in humans and animals. Most reports indicate that giardias do not invade the mucosa of the small intestine to a significant extent, and thus disease appears to follow directly from attachment (23, 24). The mechanism of attachment of trophozoites to intestinal cells has not been established definitively. Evidence supports roles for the ventral disk, which is considered a specific attachment organelle (5, 11); trophozoite contractile elements (5, 8); hydrodynamic and mechanical forces (12); and lectin-mediated binding (5, 18). Despite the apparent importance of attachment of trophozoites to intestinal epithelial cells for disease and perhaps for the initiation and maintenance of infection, this process has been studied little because of a lack of an appropriate in vitro model. We report here a simple, convenient, inexpensive, rapid, and reproducible method for quantitation of binding of radiolabeled trophozoites to IEC-6 cells. IEC-6 cells are a continuously cultured cell line derived from the rat small intestine that retains morphologic, immunologic, and functional characteristics of native cells (3, 20, 21).

MATERIALS AND METHODS

Giardias. Trophozoites of *Giardia intestinalis* WB, a strain derived from a human duodenal aspirate specimen and obtained from the American Type Culture Collection (Rockville, Md.), were used unless stated otherwise. In some experiments, the Portland-1 strain (American Type Culture Collection) and strain N-0782 were used; both strains were recovered from humans. The latter strain was kindly provided by John I. Bruce (Center for Tropical Disease, University of Lowell, Lowell, Mass.).

Trophozoites were cultivated in TYI-S-33 medium supplemented with bile at 37°C in either 10-ml glass tubes or 550-ml glass roller bottles (4, 15). When cultures were in logarithmic growth phase, trophozoites unattached to glass were removed by decanting the medium and then discarded. Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) was added to the culture vessel, which was then chilled in an ice bath for 10 min to detach trophozoites from glass. After two washes in Hanks balanced salt solution (300 × *g* for 10 min at 25°C), the trophozoites were resuspended in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 5% heat-inactivated (60°C) fetal calf serum (HyClone Laboratories, Logan, Utah), penicillin (100 U/ml) (Sigma Chemical Company, St. Louis, Mo.), and streptomycin (100 μg/ml) (Sigma), hereafter referred to as DMEM. Trophozoites were counted in a hemacytometer and adjusted to the desired concentration with DMEM. Trophozoites were used for experiments only when they were more than 95% viable as assessed by motility and exclusion of trypan blue.

Radiolabeling trophozoites with [^{3}H]thymidine. Fifty millicuries of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, 84.1 μCi/mmol; New England Nuclear Research Products, Boston, Mass.) was added during the logarithmic growth phase to 10 ml of medium in each glass tube, or 1.5 Ci was added to 550 ml of medium in each roller bottle, and the tubes and bottles were incubated for 24 h more. Trophozoites were then washed repeatedly in Hanks balanced salt solution until the radioactivity in the wash was less than 3% of that of the pelleted organisms, and then they were resuspended in DMEM.

IEC-6 cells cultivation. Monolayers of IEC-6 cells (American Type Culture Collection) were cultured routinely in DMEM in tissue culture flasks (no. 3150; Costar, Cambridge, Mass.) at 37°C in 5% CO_2 in air (3, 20, 21). For

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experiments, IEC-6 cells were trypsinized and then inoculated into 8-well Lab-tek tissue culture chambers (0.79 cm^2 per well; Lab-tek Products, Miles Laboratories, Naperville, Ill.), 24-well tissue culture trays (Costar), or 96-well tissue culture trays (Costar). The cells were grown on 12- and 5-mm-diameter glass coverslips (Bellco Glass Inc., Vineland, N.J.) in 24- and 96-well tissue culture trays, respectively. The cultures were incubated until the monolayers were completely confluent, when they were used for experiments.

Binding assay. Trophozoites were added to monolayers of IEC-6 cells and incubated at 37°C in 5% CO_2 for different periods of time. Lab-tek chambers were washed to remove unbound trophozoites. For experiments with glass coverslips, the wells were washed twice, and the coverslips were dipped twice into normal saline to remove unbound giardias. Further washing in normal saline did not remove more trophozoites. The coverslips were then placed in scintillation vials that contained Safety-Solve (Research Products International Corp., Mount Prospect, Ill.), and radioactivity was measured with a Mark III beta counter (Searle Analytic Inc., Elk Grove Village, Ill.). Counting was typically for 2 min, and counts were expressed as disintegrations per minute. Trophozoites often adhered to the exposed glass on the underside of the coverslip. It was essential to remove the giardias attached to the glass with a damp Kimwipe (Kimberly Clark Corp., Roswell, Ga.). For Lab-tek chambers, the wells were cut with glass cutters and the radioactivity of each well was measured with a scintillation vial. Each experiment that used radiolabeled trophozoites was performed with at least three replicates. In addition, the accuracy of each experimental point was confirmed by a Giemsa-stained replicate (Harleco, Gibbstown, N.J.).

In some experiments, cytochalasins A, B, and D (Sigma) and colchicine (Sigma) were incubated with trophozoites for 30 min and then added to the tissue culture chamber so that the final concentration of drug was less than 1 μg/ml. The trophozoites were coincubated with IEC-6 cells for 2 h and then assayed for the number of bound trophozoites. The cytochalasins were dissolved in dimethyl sulfoxide (Sigma) for a stock concentration of 1.0 mg/ml. Further dilutions were done with DMEM; control cultures contained equivalent amounts of dimethyl sulfoxide. Preliminary experiments established that these agents did not affect the viability of trophozoites, as assessed by trypan blue dye exclusion.

In some experiments, IEC-6 cell monolayers or trophozoites were fixed with 0.25% glutaraldehyde in 0.15 M NaCl for 15 min and washed once each with 0.15 M glycine in water and Hanks balanced salt solution (14). The fixed IEC-6 cells or trophozoites were then incubated at 37°C in 5% CO_2 with viable trophozoites or IEC-6 cells to determine whether living cells were needed for binding. For experiments to assess the effects of divalent cations, EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Sigma) were incubated with trophozoites for 1 h at a concentration of 10 mM. Trophozoites were resuspended in fresh medium without chelator and then incubated with IEC-6 cell monolayers for 2 h. Trophozoites were coincubated with IEC-6 cells in the foregoing experimental protocols. To assess the avidity of binding of trophozoites to IEC-6 cells, monolayers were incubated for 1 h on a rotational shaker (Orbital shaker; Bellco Glass) at 75 rpm.

Electron microscopy. Monolayers of IEC-6 cells incubated with trophozoites for 30 min on plastic coverslips were fixed in 2% glutaraldehyde in sodium cacodylate buffer for 24 h at 4°C. The coverslips were then postfixed in 1% osmium tetroxide for 1 h. After carbon coating, scanning electron microscopy was done with a Cambridge Mark IIA scanning electron microscope. For transmission electron microscopy, the specimen was stained en bloc with 2% uranyl acetate, dehydrated in graded alcohol, and embedded in Spurr's low-viscosity resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 electron microscope.

Statistics. Student's *t* test was used to assess differences, with a P of <0.05 considered significant. Three to four replicates were used for each experimental point. Results representative of a minimum of three experiments are presented for the WB strain, and results representative of two experiments are presented for the Portland-1 and N-0782 strains.

RESULTS

Electron microscopy. Scanning electron microscopy showed the IEC-6 cells as a confluent monolayer (Fig. 1). The IEC-6 cell surface had variable numbers of small microvilli, and none of the cells had a brush border as seen on mature intestinal-surface epithelial cells. Numerous trophozoites were attached to the surfaces of the cells, with no apparent predilection for cells with more microvilli. The ventral disks of most giardias were in contact with the monolayer. By transmission electron microscopy (Fig. 2), most IEC-6 cells appeared healthy and intact, although occasional degenerated cells were seen. The trophozoites attached to the cell surface apparently by the edges of the ventrolateral flanges. The plasma membrane and, occasionally, the nucleus profile were distorted by flanges pinching onto the cell surface.

Binding of giardias to IEC-6 cell monolayers. The number of trophozoites that attached to IEC-6 cells varied directly with the number added to the monolayer (Fig. 3), although the proportion that attached decreased with larger inocula. Inspection of the monolayers at these high concentrations before the washes showed that the trophozoites were hyperconfluent. Counting trophozoites attached to IEC-6 cells in Giemsa-stained duplicate monolayers with the light microscope revealed a similar relationship of the size of the inoculum of trophozoites to the number of attached organisms (Fig. 3B).

Trophozoites remained attached to the IEC-6 cells for at least 24 h (Fig. 4) and up to 48 h in a single experiment (not shown). In parallel experiments, giardias were more than 95% viable in DMEM for at least 24 h, as assessed by motility and trypan blue dye exclusion. The IEC-6 cell monolayers were intact when assessed by either indirect light microscopy or Giemsa staining at 24 h. The loss of a few IEC-6 cells from the monolayer was evident when large numbers of trophozoites, sufficient to form a hyperconfluent layer over the IEC-6 cell monolayer, were added. Similar relationships between the number of attached trophozoites and the time of incubation were observed in three experiments with Giemsa-stained monolayers. In a typical experiment, the numbers of attached trophozoites (organisms per 10 oil-immersion fields) at 1, 2, 4, 20, and 24 h of coincubation were 62, 65, 40, 73, and 63, respectively.

No substantial differences were observed in the results obtained with the foregoing experimental protocols when Lab-tek tissue culture chambers, 24-well plates, and 96-well tissue culture trays were used (data not shown).

Effects of cytochalasins, colchicine, and glutaraldehyde fix-

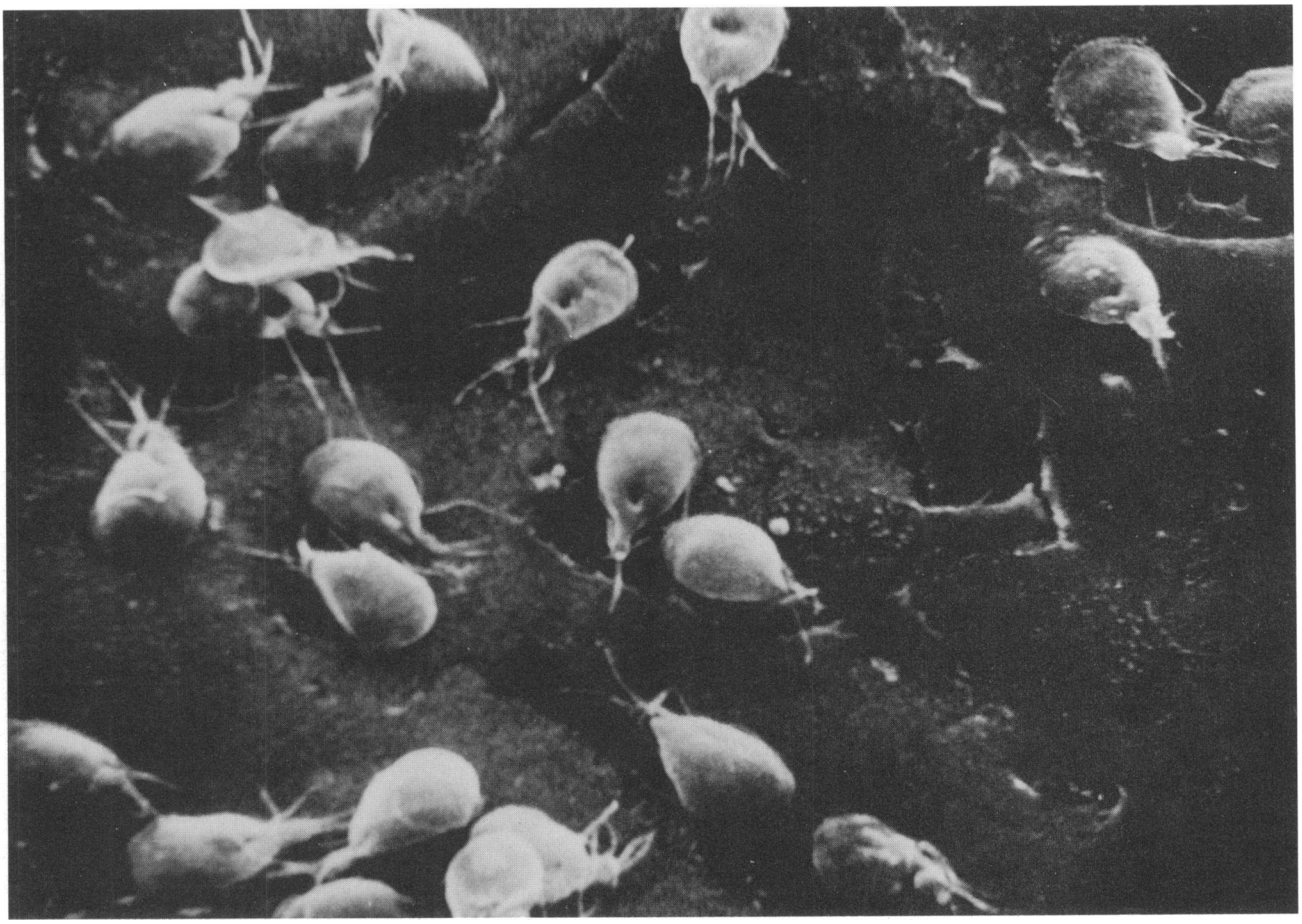

FIG. 1. Scanning electron micrograph showing confluent monolayer of IEC-6 cells with surface microvilli. Most giardia trophozoites are situated on the cell surface, with the ventral disks in contact with the cell. Magnification, ×5,900.

ation. Incubation of trophozoites with cytochalasins A, B, and D for 30 min before addition to monolayers significantly inhibited attachment to IEC-6 cells to about 20% of control values (Fig. 5). No marked differences were apparent among the results obtained with different cytochalasins. Colchicine, in contrast, had no effect on attachment. Counting attached trophozoites in Giemsa-stained duplicate monolayers produced parallel results with cytochalasins and colchicine. Compared with a control value of 312 ± 16 trophozoites per 10 oil-immersion fields ± standard error of the mean, cytochalasin A (5 and 20 μg/ml), cytochalasin B (5 and 20 μg/ml), cytochalasin D (5 μg/ml), and colchicine treatments resulted in 22 ± 4, 14 ± 8, 72 ± 12, 50 ± 10, 50 ± 4, and 285 ± 7 attached trophozoites, respectively ($P < 0.05$ for all treatments except colchicine).

Glutaraldehyde fixation of IEC-6 cells diminished but did not abolish attachment, which was approximately half that of controls (Fig. 5). Glutaraldehyde fixation of trophozoites reduced attachment to (17 ± 10)% (mean ± standard error of the mean) of control values ($P < 0.005$) in three experiments.

Effects of EDTA, EGTA, rotational shaking, and temperature on attachment. Preincubation of trophozoites with EDTA and EGTA significantly inhibited trophozoite attachment to IEC-6 cells to (24 ± 18)% and (26 ± 12)% of control values, respectively (mean ± standard error of the mean of three experiments). In one experiment, incubation at 4°C significantly reduced attachment to 4% of the value for controls incubated at 37°C. The use of a rotational shaker significantly reduced attachment to (60 ± 3)% (mean ± standard error of the mean) of the control values in three experiments. In two experiments each with the rotational shaker, EDTA, EGTA, and glutaraldehyde fixation of trophozoites significantly reduced attachment to values of (11 ± 4)%, (26 ± 1)%, and (12 ± 1)%, respectively, and incubation at 4°C in one experiment significantly reduced attachment to 7% of the control values.

Results with Portland-1 and N-0728 strains. Increasing the number of giardias added to IEC-6 cells resulted in attachment of more giardias, and cytochalasins A and B significantly inhibited attachment (Tables 1 and 2). These results parallel those obtained with the WB strain.

DISCUSSION

These experiments show that axenically cultured giardia trophozoites attach firmly to IEC-6 cells in vitro. Other investigators have shown that manipulation of temperature, divalent cation concentration, or actin-myosin function affects binding of giardia trophozoites to glass or plastic (6, 9, 25). We have shown that similar manipulations similar-

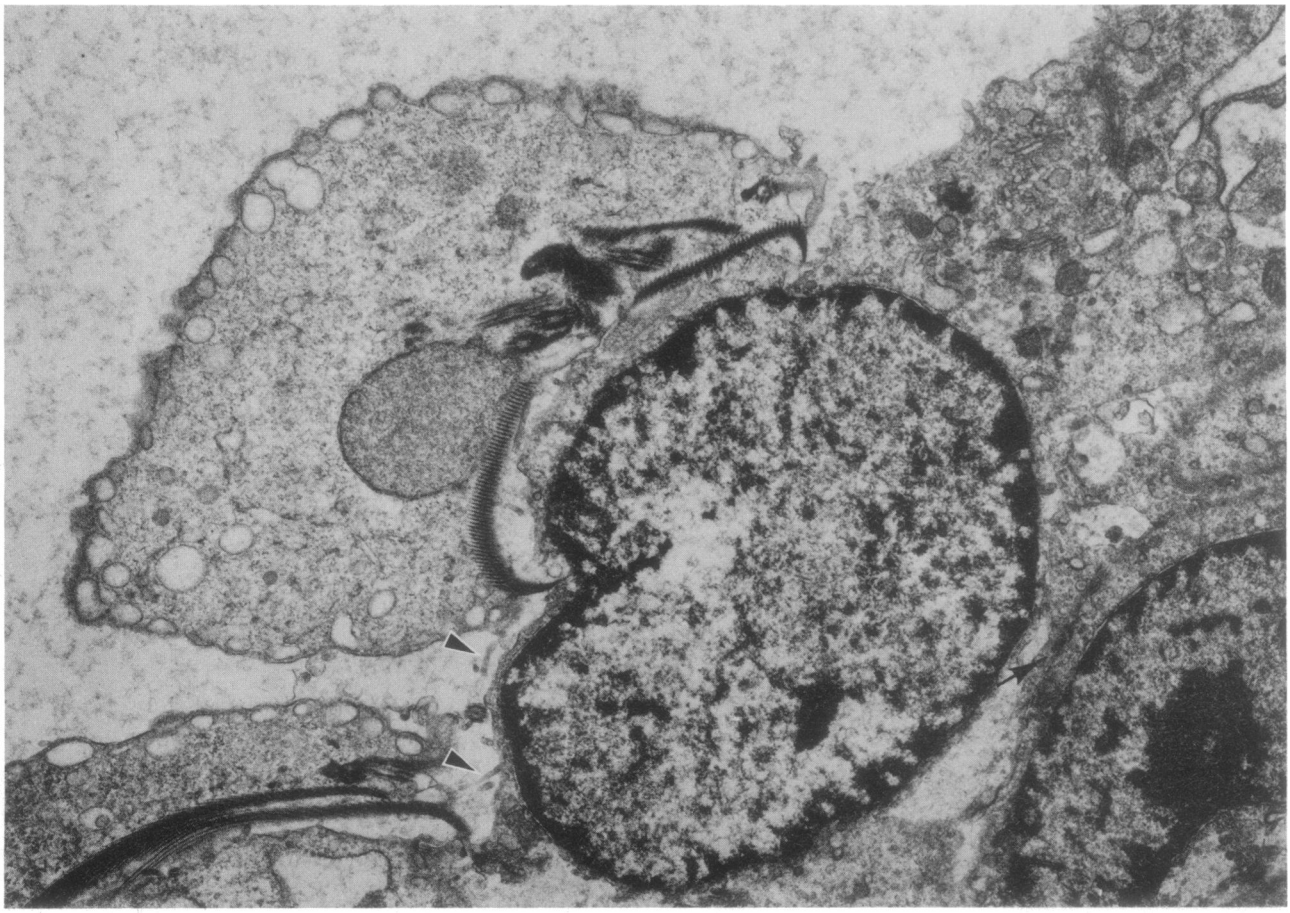

FIG. 2. Transmission electron micrograph of IEC-6 cell monolayer with attached trophozoites. The IEC-6 cells show intercellular junctions (arrow) and surface microvilli (arrowheads). The flanges of the trophozoite have indented the IEC-6 cell surface plasma membrane and appear to have deformed the nuclear membrane. Magnification, ×11,600.

ly influence binding of trophozoites to cells. Noteworthy is that similar results in experiments of like design were obtained in studies of binding of *Entamoeba histolytica* to cells (16).

Attachment can be assayed with coverslips in 24- and 96-well tissue culture trays with [^{3}H]thymidine-labeled trophozoites. However, the use of eight-well Lab-tek tissue culture chambers was most convenient and straightforward.

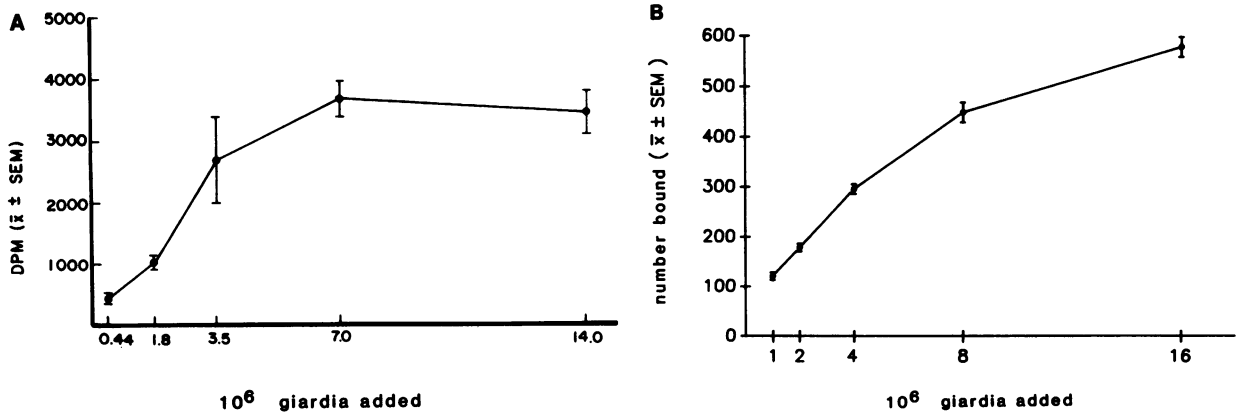

FIG. 3. Effect of adding increasing numbers of [^{3}H]thymidine-labeled trophozoites to IEC-6 cell monolayers in a 24-well tissue culture plate. Radioactivity was measured after 2 h of incubation of trophozoites with the monolayers. (B) Effect of adding increasing numbers of trophozoites to IEC-6 cell monolayers in Lab-tek tissue culture chambers, as assessed by counting trophozoites with light microscopy in duplicate Giemsa-stained monolayers after 2 h of coincubation.

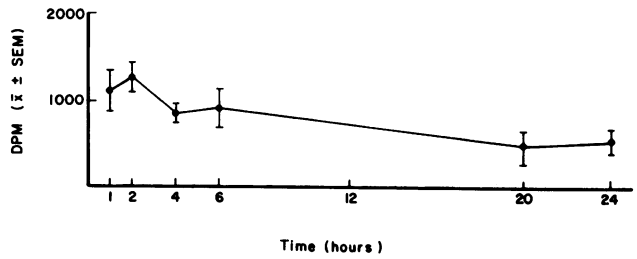

FIG. 4. Effect of length of incubation of giardias with IEC-6 cells. The average of three experiments is shown. A total of 1.8×10^6 [^{3}H]thymidine-labeled trophozoites were added to each well of a 24-well tissue culture plate, and radioactivity was determined 2 h later.

TABLE 1. Effect of increasing the number of radiolabeled trophozoites incubated with IEC-6 cells on attachment[a]

No. of giardias added (10^6)	dpm ± SEM		
	WB	Portland-1	N-0728
0.7	231 ± 10	364 ± 69	607 ± 35
1.4	299 ± 45	997 ± 137	1,376 ± 193
2.8	579 ± 7	1,741 ± 76	5,240 ± 783

[a] Trophozoites were added to IEC-6 monolayers in eight-well Lab-tek tissue culture chambers for 2 h and washed free of unattached trophozoites, and then radioactivity was measured. Incorporation of radiolabel varied among strains and from experiment to experiment, and differences in counts among strains do not indicate adherence of more trophozoites.

This in vitro model may be useful for investigating the mechanisms by which trophozoites attach to intestinal cells (i.e., initiate infection) and possibly induce disease (i.e., affect intestinal-cell function). Furthermore, this model may facilitate the evaluation of antimicrobial agents that act by either prevention of attachment or induction of detachment of organisms by mechanisms other than production of cell toxicity. An obvious limitation of this model as well as others is that the experiments are conducted under aerobic conditions needed for viability of the IEC-6 cells, whereas giardias are aerotolerant anaerobes. Although trypan blue dye exclusion and motility indicated viability of trophozoites, subtle metabolic derangements induced by exposure to air may have played a critical role in inhibiting attachment in some experiments. Limited experiments in which trophozoites were incubated with IEC-6 cell monolayers for 2 h under anaerobic conditions produced in a Gas-Pak system (BBL, Cockeysville, Md.) showed no difference in attachment of trophozoites under aerobic and anaerobic conditions (data not shown).

Previous studies of attachment of giardia trophozoites used glass or plastic as the substrate (6, 9, 25), enterocytes isolated from rodents (13, 18), segments of surgically isolated murine intestine inoculated with trophozoites from which villi were then removed for evaluation of attachment (14), or continuously cultured cell lines, e.g., Madin-Darby canine kidney (MDCK) cells (2) and fibroblasts (22). Glass or plastic substrates are convenient but are dissimilar to in vivo substrates. The use of freshly isolated enterocytes or villi is tedious and labor-intensive, and these cells may be difficult to manipulate experimentally. Continuously cultured cell lines are convenient, relatively easily manipulated, and may exhibit phenomena similar to those occurring in vivo. IEC-6 cells were used because they are derived from rat small intestine; retain morphologic (e.g., microvilli and tight junctions), enzymatic, and immunologic characteristics of native rat small-intestinal crypt cells; and behave similarly to crypt cells in the presence of factors that regulate cell proliferation in vitro (3, 17, 20, 21).

Although IEC-6 cells retain some of the characteristics of native intestinal crypt cells, they appear by light microscopy to grow in a nonpolarized manner typical of undifferentiated epithelial cells. Many of the features indicative of small-intestinal crypt cell origin are derived from immunologic experiments and transmission electron microscopy. We did not determine whether the expression of those features characteristic of native intestinal crypt cells is actually playing a role in the adherence of giardia trophozoites to IEC-6 cells. Furthermore, some data indicate that giardia trophozoites attach preferentially to crypt cells in mice (19), but whether trophozoites attach preferentially to crypt or villus cells in humans is not clearly established. There are major differences in crypt cells and villus cells, e.g., with respect to lectin binding and enzymatic activity, which may bear on the appropriateness of IEC-6 cells for an in vitro model of binding of trophozoites (1, 21). More investigations are needed to assess the appropriateness of IEC-6 cells for

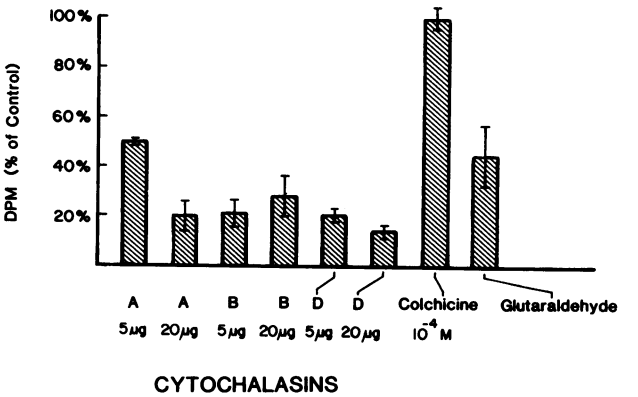

FIG. 5. Effects of cytochalasins A, B, and D and colchicine treatment of trophozoites and glutaraldehyde fixation of IEC-6 cells on attachment of [^{3}H]thymidine-labeled trophozoites to IEC-6 cell monolayers in 24-well plates. Trophozoites were incubated with monolayers for 2 h. See text for details of different treatments. $P < 0.05$ for all differences of treatment versus the control, except for colchicine.

TABLE 2. Effect of cytochalasin treatment on attachment of radiolabeled trophozoites to IEC-6 cells[a]

Treatment	dpm ± SEM		
	WB	Portland-1	N-0728
Cytochalasin A	311 ± 59	422 ± 55	176 ± 2
Control	992 ± 187	1,914 ± 147	1,045 ± 254
Cytochalasin B	ND[b]	166 ± 133	957 ± 88
Control	ND	2,003 ± 176	1,508 ± 164

[a] Trophozoites were incubated with cytochalasin for 30 min, diluted so that the concentration of cytochalasin was less than 1 μg/ml, and incubated with IEC-6 monolayers for 2 h in eight-well Lab-tek tissue culture chambers, and then radioactivity was measured. A total of 1.36×10^6 trophozoites were added to each well. All differences among cytochalasin treatments and controls were significant ($P < 0.05$).

[b] ND, not done.

an in vitro model of giardiasis, especially in comparison with other cell lines.

In view of the different models and experimental procedures used to study attachment of giardias, it is perhaps not surprising that experimental results do not always agree among studies. Feeley and Erlandsen (6) found that cytochalasin B reduced attachment of giardia trophozoites that had been scraped from the small intestines of infected rats to polystyrene petri dishes, whereas colchicine had no effect. Our results were similar and indicate a role for the actin-myosin system in attachment, with little evidence of a role for the microtubule system. Both actin and microtubules have been found in the periphery of the ventral disk (7). In contrast, Gillin and Reiner (9) found that cytochalasins B, C, and D had little effect on attachment of axenically cultured trophozoites to glass. We found that cytochalasins differed little in inhibiting attachment, which suggests that cytochalasins inhibit attachment by interfering with the actin-myosin system rather than by other mechanisms which are affected differentially by the cytochalasins (10). Inge and colleagues (13), in a model that used freshly isolated enterocytes, found that cytochalasin B and removal of divalent cations from the incubation medium did not inhibit attachment. In a previous publication with a different model (5), Farthing et al. found that divalent cations promoted erythrocyte rosette formation induced by giardia trophozoites. Gillin and Reiner (9) found that chelation of magnesium and calcium ions did not significantly inhibit trophozoite attachment to glass, whereas Feely and Erlandsen (6) found that calcium was important for attachment to polystyrene petri dishes.

Incubation of giardias with cell lines may be useful for investigating the pathogenesis of diarrhea. IEC-6 cell toxicity due to incubation with trophozoites was not usually observed when assessed by light microscopy of Giemsa-stained monolayers. In some experiments, IEC-6 cells occasionally detached from the monolayer when large numbers of trophozoites were added. Transmission electron microscopy suggested that giardias may induce direct cell damage, since some IEC-6 cells with trophozoites attached to their surfaces appeared degenerate. Furthermore, the trophozoites appeared to indent into the IEC-6 cell membrane and even to deform the nucleus. The electron micrographs were obtained after only 30 min of coincubation of giardias with the IEC-6 cells, and possibly more damage might be evident with longer coincubations.

Reports conflict on the cell-damaging capabilities of trophozoites (reviewed in reference 2). Two reports are of particular interest in the context of our results. Radulescu et al. (22) found that giardia trophozoites were very toxic to fibroblasts when coincubated for 24 h. However, as pointed out by Chavez et al. (2), it was not clear in their report whether the medium was toxic to the fibroblasts. Our experiments do not show the degree of cytotoxicity observed by Radulescu et al. (22). Chavez et al. (2), using MDCK cells, a transporting epithelial cell line, mounted as monolayers in an Ussing chamber, found no change in transepithelial resistance when the monolayers were coincubated with trophozoites for up to 48 h. Chavez et al. (2) documented attachment of trophozoites to MDCK cells by electron microscopy, but the number of attached giardias was not clearly stated in the electrophysiology experiments. More experiments are needed to determine whether incubation with trophozoites or trophozoite products influences cell viability, function, or proliferative capacity.

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