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Contact-Dependent Transfer of the Galactose-Specific Lectin of *Entamoeba histolytica* to the Lateral Surface of Enterocytes in Culture

ANCY LEROY,[1] GEORGES DE BRUYNE,[2] MARC MAREEL,[2] COMMUNIAN NOKKAEW,[3] GORDON BAILEY,[3] AND HANS NELIS[1]*

Laboratory of Pharmaceutical Microbiology, Department of Pharmaceutical Analysis, University of Ghent,[1] and Laboratory of Experimental Cancerology, Department of Radiotherapy, Nuclear Medicine and Experimental Cancerology, University Hospital,[2] B-9000 Ghent, Belgium, and Department of Microbiology and Immunology, Morehouse School of Medicine, Atlanta, Georgia 30310[3]

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In a study to investigate early interactions of *Entamoeba histolytica* with epithelial cell monolayers, we found that a monoclonal antibody (MAb), CD6, against an ameba surface antigen recognized the lateral surface of epithelial cells after coculture with trophozoites. Display of the CD6 antigen on the epithelial cells necessitated contact with active trophozoites. It was found neither at 4°C, nor with prefixed trophozoites, nor with trophozoite-conditioned media, nor when a filter prevented direct contact. Monolayers exposed to amebic sonicates or detergent lysates showed random immunostaining. Access to the antigenic site was limited, as immunostaining occurred predominantly with subconfluent monolayers. CD6 epithelial cell binding was first observed after 5 min of coculture; trophozoite-mediated target cell lysis was not detected until 15 min following coculture. Epithelial cell immunostaining occurred with some other ameba-specific antibodies but not with MAbs raised against the 170-kDa subunit of the galactose–*N*-acetylgalactosamine (Gal/GalNAc)-specific lectin. The CD6 MAb as well as some other ameba-specific antibodies immunoprecipitated from trophozoite lysates the same bands as the MAbs against the cysteine-rich domain of the 170-kDa subunit of the Gal/GalNAc-specific lectin. Why the latter MAbs failed to stain epithelial cells in the vicinity of attached trophozoites is presently unknown. We concluded that *E. histolytica* trophozoites transferred the intact amebic Gal/GalNAc-specific lectin or a portion of it to the lateral surface of epithelial cells. This juxtacrine transfer preceded killing of target cells.

Amebiasis is caused by ingestion of *Entamoeba histolytica* cysts that convert to trophozoites inside the lumen of the gut. Trophozoites colonize the lumen and occasionally invade the colon mucosa, leading to colonic ulceration (13). The molecular mechanism of mammalian target cell destruction by the *E. histolytica* trophozoites is not well defined. This process is proposed to include secretagogue-mediated depletion of the enteric mucus (45) and adhesion of trophozoites to the surface of the enterocytes (28, 33), followed by target cell lysis (14). These cytopathic effects have been ascribed to a direct contact between trophozoites and mammalian cells (32), although specific proteolytic enzymes may also participate in this process (37).

The Gal/GalNAc-specific lectin, first described as a Gal NAc-inhibitable amebic adhesin by Ravdin et al. (36), has been implicated in early trophozoite-enterocyte interactions (4, 19, 21, 29), as it plays a role in trophozoite adhesion (30) and in antigen-specific cell-mediated immunity (31). Other molecules of interest for the early steps of trophozoite invasion include a 220-kDa lectin sensitive to functional neutralization by *N*-acetyl-D-glucosamine (39), a 25-kDa serine-rich *E. histolytica*-specific protein (42), and the amphipathic amebapore 4- to 5-kDa proteins (14).

We became interested in amebiasis as a model for cancer metastasis (15). Our initial intent was to investigate the motility of trophozoites when confronted with an epithelial cell monolayer. To detect trophozoites, cocultures were immunostained with the monoclonal antibody (MAb) CD6 recognizing an unidentified trophozoite surface antigen as well as with other antibodies, some of which recognized the 170-kDa Gal/GalNAc-specific lectin subunit. The present report describes binding of MAb CD6 to the surface of epithelial cells in the vicinity of attached trophozoites and the identification of the CD6 antigen as the Gal/GalNAc-specific lectin.

MATERIALS AND METHODS

Trophozoites. Trophozoites of *E. histolytica* HM1:IMSS (provided by H. Vermeersch, Laboratory of Pharmaceutical Technology, University of Ghent, Ghent, Belgium) were grown axenically at 35.5°C in 15-ml screw-cap tubes with TYI-S-33 medium (6), supplemented with 500 μg of piperacillin per ml and 125 μg of amikacin per ml. Trophozoites were harvested at the end of the logarithmic growth phase (72 h) by chilling at 4°C. They were concentrated at 200 × *g* for 5 min and used immediately. For some experiments, freshly harvested trophozoites were fixed for 20 min in 3% paraformaldehyde (PF), washed in phosphate-buffered saline (PBS; pH 7.4) containing 0.9 mM Ca^{2+} and 0.334 mM Mg^{2+} (PBS^{D+}), and quenched for 10 min with NH_4Cl (50 mM in PBS^{E} [Eisen formulation]). Thereafter, they were handled in the same way as live trophozoites. Conditioned medium was prepared from (i) logarithmic-growth-phase cultures in TYI-S-33; (ii) TYI-S-33, with or without serum, that had been incubated for 1 h with the total number of cells from an equal volume of a logarithmic-phase culture; and (iii) cocultures of trophozoites with mammalian cells as described below. Trophozoite cell extracts were prepared by one of the following methods: (i) intermittent sonication (Vibra Cell VC50; Sonics & Materials, Danbury, Conn.) of 2×10^6 live trophozoites in 0.5 ml of PBS^{D+} on ice for a total of 45 s or (ii) vortexing of 2×10^6 live trophozoites at 4°C in 150 μl of lysis buffer (50 mM Tris, 1 M NaCl, 1% Triton X-100) followed by centrifugation at 16,000 × *g* for 5 min.

* Corresponding author. Mailing address: Laboratory of Pharmaceutical Microbiology, Department of Pharmaceutical Analysis, University of Ghent, Harelbekestraat, 72, B-9000 Ghent, Belgium. Phone: 32-9-221 31 08 or 32-9-221 89 51, ext. 234. Fax: 32-9-221 45 33.

Human cell lines. Enterocyte cell lines, derived from human colon cancers, used were Caco-2 (HTB37; American Type Culture Collection [ATCC], Rockville, Md.), LoVo (CCL 229; ATCC), HCT-8/S (a spread variant from HCT-8) (CCL 244; ATCC), COLO320DM (CCL 220; ATCC), and HCT-8/R (a round-cell variant isolated from an HCT-8 culture by F. Van Roy, University of Ghent, Ghent, Belgium). Human epithelial breast MCF-7/6 cells as well as MRC-5 (CCL 171; ATCC) and ICIG-7 (2) fibroblasts were included as nonenterocytic counterparts. The dog kidney cell line MDCK (CCL 34; ATCC) was used because of its property of forming polarized monolayers on tissue culture inserts (10). Before storage in our liquid nitrogen bank, the identities of all cell lines were checked with cell lineage markers. All culture media were purchased from Gibco Europe, Ghent, Belgium. Caco-2 and MDCK cells were grown in Dulbecco's modified Eagle's medium with 0.05% L-glutamine, supplemented with nonessential amino acids and 15% and 10% fetal bovine serum (FBS), respectively. HCT-8 and COLO320DM were cultured in RPMI 1640 with 10% FBS, supplemented with 1 mM sodium pyruvate and 0.05% L-glutamine, respectively. MRC-5 and ICIG-7 cells were maintained in Eagle's minimal essential medium containing Earle's salts and 1% nonessential amino acids (100×). LoVo cells were grown in HAM F12 with 20% FBS. MCF7/6 cells were cultured in Dulbecco's modified Eagle's medium-HAM F12 (50:50), both supplemented with 10% FBS and 0.05% L-glutamine. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added to all culture media. Cells were maintained in 25-cm^2 tissue culture plastic vessels (Nunc, Roskilde, Denmark) at 37°C in 10% CO_2 for Caco-2 and MCF7/6 cells or in 5% CO_2 for the other cell types, in humidified incubators. Fixed monolayers were used in some experiments. To this end, cultures were treated as described above for fixed trophozoites. To simulate the uncovering of putative epithelial epitopes by the lytic activities of trophozoites, monolayers were treated in one of the following ways: with trypsin (0.1%; Gibco BRL) for 1 to 10 min; with neuraminidase (0.4 U/ml; Sigma) for 15 to 60 min (5); or with 4-methylumbelliferyl-β-D-xyloside (0.5 to 5 mM; Sigma) for 2 to 22 h (48).

Cocultures. The vertebrate cells were cultured for 2 and 4 days in Lab-Tek 8-Chamber Slides (Permanox; Nunc) to obtain subconfluent and confluent monolayers, respectively. Suspensions of 3×10^4 freshly harvested or fixed trophozoites in 20 μl of TYI-S-33 medium were seeded onto monolayers in a ratio of one trophozoite to four vertebrate cells, and cocultures were further incubated under the conditions used for vertebrate cell stock cultures. Similar cocultures were made by using 60-mm^2 polycarbonate tissue culture insert filters (pore size, 8.0 μm; Nunc) in 9.3-cm^2 six-well dishes and adding trophozoites to the upper chamber. For the latter cultures the transepithelial electrical resistance (TER) of the monolayer was measured by using a Millicell electrical resistance system (Millicell-ERS; Millipore, Bedford, Mass.) as prescribed by the manufacturer. For cocultures at 4°C, the monolayer was precooled and all further manipulations were done at 4°C, as described above. To test interactions mediated by secretory products, enterocytic monolayers on tissue culture insert filters were explanted onto trophozoites cultured for 1 h on the bottom of the six-well dishes. To inhibit lectin-mediated interactions (3, 33), experiments were done with the following sugars (Sigma, St. Louis, Mo.): D-(+)-galactose (100 mM); β-lactose (100 mM); *N*,*N*′,*N*″-triacetylchitotriose (40 mM); *N*-acetyl-D-galactosamine (100 mM); asialofetuin (1%); and heparin (200 IU/ml). To this end, trophozoites were pretreated (20 min at 4°C) and cocultures (60 min at 37°C) were done in the presence of the sugar residue. To inhibit a presumed proteolytic activity, trophozoites were treated before (for 20 min) and during (for 15 to 60 min) coculture with one of the following inhibitors (all from Sigma): aprotinin at 1 to 10 μg/ml; soybean trypsin inhibitor at 100 μg/ml; *trans*-epoxysuccinyl-L-leucylamido (guanidino) butane at 200 μM; *N*-ethylmaleimide at 0.2 to 2 mM; *para*-hydroxymercuribenzoate (pHMB) at 1 to 10 mM; iodoacetamide (IA) at 0.5 to 5 mM; phenylmercuriacetate (PMA) at 1 to 10 mM; phenylmethylsulfonyl fluoride at 1 mM; or α2-macroglobulin at 1.5 mg/ml (1, 22, 24). All cocultures were done in duplicate with controls containing fixed trophozoites or lacking trophozoites. Relevant experiments were repeated at least once.

Antibodies. MAbs CD6, CC3, CB4, and CH10 were prepared from mice according to standard procedures (11) by using whole trophozoites injected intraperitoneally as an antigen. Hybridomas containing surface reactive antibodies (detected by immunofluorescence as described below) were cloned, and antibodies were produced in mouse ascites. To prepare polyclonal antibodies, rabbits were subcutaneously and intramuscularly injected with 7.5×10^5 heat-inactivated (1 h at 56°C) whole trophozoites in complete Freund's adjuvant, followed by four boosters of 2×10^6 heat-inactivated trophozoites at 2-week intervals. Immune sera (coded 1553, 1554, and 1556) were harvested at the time of the third booster. Mouse monoclonal immunoglobulin G2a (IgG2a) (FP10, FP14, and FP21) and IgG2b (30B) antibodies recognizing, respectively, a 29-kDa antigen and a 96-kDa cytoplasmic antigen (7, 44) were kindly provided by B. Torian (Idaho State University, Pocatello). Mouse monoclonal IgG2b (7F-4, H8-5, and 1G-7) and IgG1 (3F-4) antibodies and rabbit polyclonal (Shiro) antibodies against the 170-kDa heavy subunit of the Gal/GalNAc-specific lectin (26, 29, 36) were kindly provided by W. Petri (University of Virginia Medical Center, Charlottesville). Rabbit polyclonal (EH-002) and mouse IgG1 monoclonal (PL) antibodies against unidentified *E. histolytica* antigens were purchased from LMD Laboratories (Carlsbad, Calif.) and Paesel and Lorei, GmbH & Co. (Frankfurt am Main, Germany), respectively. The E-cadherin-specific rat MAb DECMA-1 was from Sigma.

Immunocytochemistry. For confocal laser scanning with a Molecular Dynamics Multiprobe 2001 microscope, trophozoites were fixed for 30 min in 5% formaldehyde and incubated at 37°C with MAb CD6 for 1 h followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (DAKO, Glostrup, Denmark) for 2 h (both diluted 1:50 in PBS with 0.1% bovine serum albumin [BSA]). Three-micrometer-thick plastic sections were made from trophozoites that were fixed, stained with the CD6 MAb and with a 5-nm-diameter gold particle-conjugated goat anti-mouse antibody (Amersham International, Amersham, United Kingdom), and subjected to silver enhancement (12) prior to being embedded in Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim, Germany). For immunocytochemistry, cocultures were fixed for 20 min in 3% PF, quenched for 10 min with NH_4Cl (50 mM in PBS^E), and permeabilized for 5 min with 0.2% Triton X-100 in PBS^E, all at room temperature with three washes in PBS^{D+} before and after each step. Cocultures were also fixed without permeabilization. Nonspecific binding sites were blocked with BSA (5% [wt/vol] in 10 mM Tris buffer, pH 7.4; 30 min). Immunostaining was carried out with MAbs (20 μg/ml) and with polyclonal antibodies (1:100) for 1 h followed by FITC-conjugated rabbit anti-mouse (RAM-FITC [dilution, 1:20]; DAKO) or goat anti-rabbit (GAR-FITC [dilution, 1:20]; DAKO) antiserum for 1 h. For double immunostaining, selected antibodies were coincubated with the E-cadherin-specific rat MAb DECMA-1 (1:1,000 dilution; Sigma). In this case the FITC-conjugated secondary antibodies were mixed with a biotinylated sheep anti-rat IgG antibody (1:50 dilution; Amersham International) and subjected to a further incubation for 15 min with streptavidin conjugated to Texas red (1:50 dilution; Amersham International). Nuclear staining was performed for 15 min with 4′,6′-diamidino-2-phenylindole (0.4 μg/ml; Serva, Heidelberg, Germany). Immunostained cultures were mounted in Glycergel (DAKO) and examined by epifluorescence microscopy (Dialux 20; Leitz, Wetzlar, Germany). Photographs were taken with the Leitz Vario-Orthomat camera system. All preparations were examined by at least three independent observers. Matched cultures without trophozoites and cocultures that were processed in the absence of the primary antibody gave no immunosignal. For confocal microscopy of cocultures, the imaging system consisted of a NIKON Optiphot-2 fluorescence microscope equipped with a 60× N.A.1.4 Plan Apo objective lens and a Bio-Rad MRC-600 confocal scanner. To evaluate cell death, live cocultures were stained with fluorescein diacetate and propidium iodide as described by Griffiths et al. (9) and examined by fluorescence microscopy.

Videography. Cocultures in six-well culture dishes (Nunc) were videorecorded with a camera (DAGE-MTI, Michigan City, Ind.) connected to an inverted microscope (Zeiss, Oberkochen, Germany) in a room at 37°C. The videorecorder (Umatic VO-585OP; Sony, Tokyo, Japan) was steered by an animation control unit (AC-580; Eos, Barry, United Kingdom) and a homemade time-date generator (46).

Radioimmunoprecipitation. ^{35}S-translabeled methionine and cysteine (Tran^{35}S-Label [70% L-methionine and 15% cysteine]; ICN Biomedicals, Lisle, Ill.) were added to trophozoite cultures (100 μCi/ml) for the last 48 h before harvest. Trophozoites (15×10^6) were lysed by resuspension in 800 μl of buffer (50 mM Tris-HCl [pH 7.4], 1 M NaCl, 1% Triton X-100, 5 mM IA, 10 mM pHMB, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml), and the lysate was cleared by centrifugation at 16,000 × *g* for 5 min. Pellets contained about 1% of the total ^{35}S-translabeled proteins from detergent-generated trophozoite lysates. Multiple 250-μl aliquots of lysate containing equal amounts of trichloroacetic acid-precipitable protein (47) were precleared by incubation with 50 μl of 50% protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) at 4°C for 30 min and centrifugation as described above. The supernatants were incubated without antibody and separately with 5 μg of CD6; 1 μg of 1553, 1554, and PL; and 0.01 μg of EH-002 for 3 h at 4°C followed by 25 μl of 50% protein G-Sepharose for 1 h at 4°C. Pellets were collected by brief centrifugation at 16,000 × *g*, washed four times with 800 μl of buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described previously (48).

RESULTS

Epithelial immunostaining by ameba-specific antibodies. To identify trophozoites in cocultures, immunostaining was initially performed with one of four available ameba-specific MAbs. Surprisingly, MAb CD6 immunostained not only trophozoites but also limited areas of the mammalian epithelial cell monolayer in the vicinity of attached trophozoites. This resulted in a characteristic honeycomb-like staining pattern at epithelial cell-cell contacts (Fig. 1A). A trophozoite-associated epithelial immunosignal was seen only when fixation of the cocultures was followed by permeabilization with Triton X-100 (compare Fig. 1A with Fig. 1B). Independent observers agreed that the areas of epithelial cell immunostaining, first seen in cocultures fixed after 5 min, increased with time of coculture and reached a maximum after 1 h. Of all ameba-specific anti-

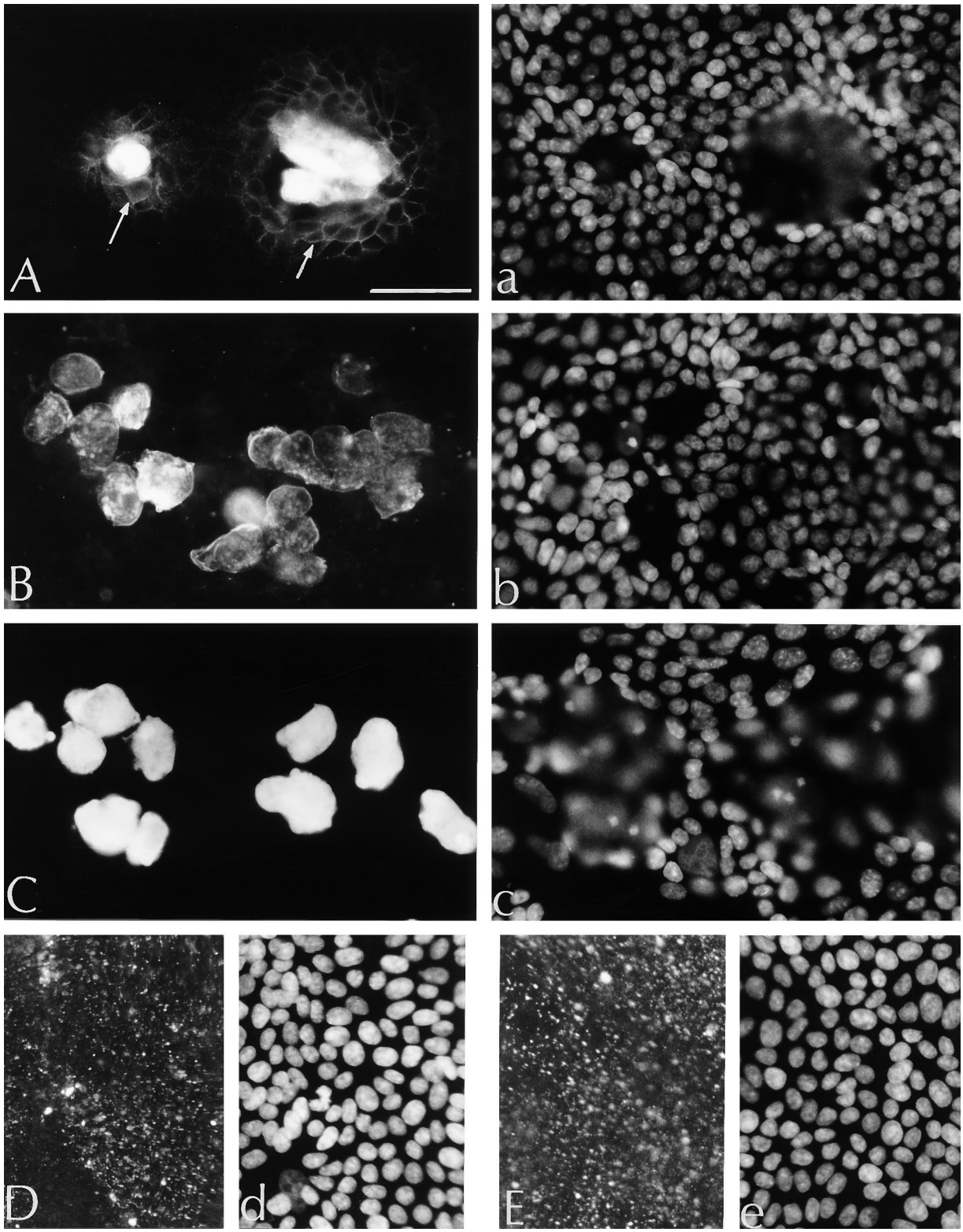

FIG. 1. Fluorescence micrographs of HCT-8/S enterocytes, fixed and immunostained after incubation for 1 h with live *E. histolytica* trophozoites (A to C) or trophozoite sonicates (D and E). All cocultures, except that for panel B, were treated with Triton X-100 following fixation. Primary antibodies were CD6 (A, B, and D) and PL (C and E). The secondary antibody was an FITC-conjugated goat anti-mouse IgG. The total cell distribution in each immunostained field was revealed by nuclear staining with 4′,6′-diamidino-2-phenylindole (a to e). Bar = 50 μm. Arrows (A) mark trophozoite-associated epithelial immunostaining.

TABLE 1. Antibodies used for the immunocytochemistry of cocultures of trophozoites and epithelial cells

Antibody or antibodies	Immunoglobulin subclass	Epithelial immunostaining	Reference or source
CD6	IgG2b	+	23
CC3, CB4	IgM	−	23
CH10	IgG	−	23
FP10, FP14, FP21	IgG2a	−	7
30B	IgG2b	−	44
7F-4, H8-5, 1G-7	IgG2b	−	27
3F-4	IgG1	−	27
Shiro	PC[a]	±[b]	27
PL	IgG1	−	PL[c]
EH-002	PC	+	LMD[d]
1553, 1554	PC	+	This study
1556	PC	−	This study

[a] PC, polyclonal antibody.
[b] ±, weak.
[c] PL, Paesel and Lorei.
[d] LMD, LMD Laboratories, Carlsbad, Calif.

bodies tested (Table 1), only three rabbit polyclonal antibodies, i.e., 1553, 1554, and EH-002, yielded the same effect as the CD6 MAb. Interestingly, several MAbs raised against the 170-kDa subunit of the Gal/GalNAc-specific surface lectin (Table 1) failed to stain the epithelium. Epithelial cell immunostaining with the CD6 MAb (Fig. 2a to d) was consistently observed

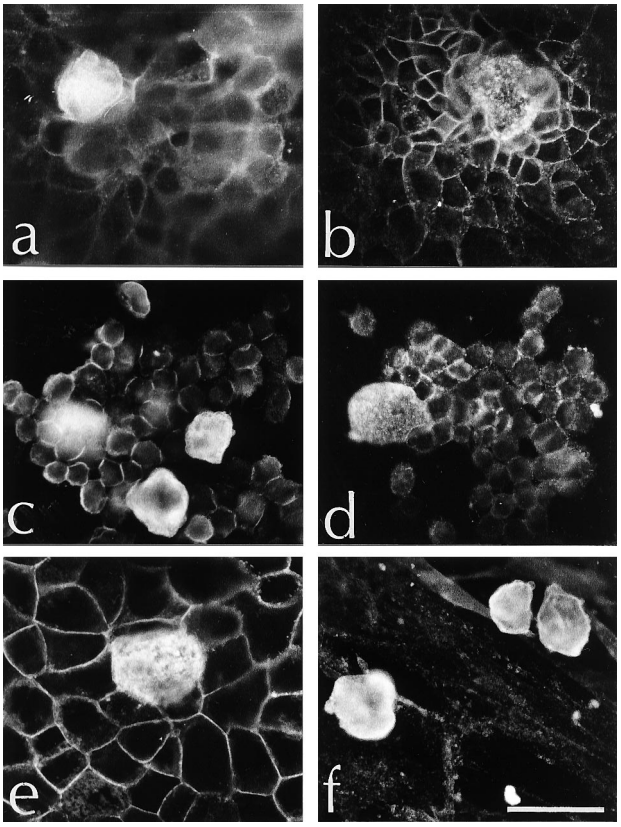

FIG. 2. Fluorescence micrographs of various cultured cell types fixed, treated with Triton X-100, and immunostained with MAb CD6 plus FITC-conjugated goat anti-mouse IgG after 1 h of coculture with *E. histolytica* trophozoites. Cell types were Caco-2 (a), HCT-8/S (b), HCT-8/R (c), COLO320DM (d), mammary MCF-7/6 cells (e), and MRC-5 fibroblasts (f). Bar = 50 μm.

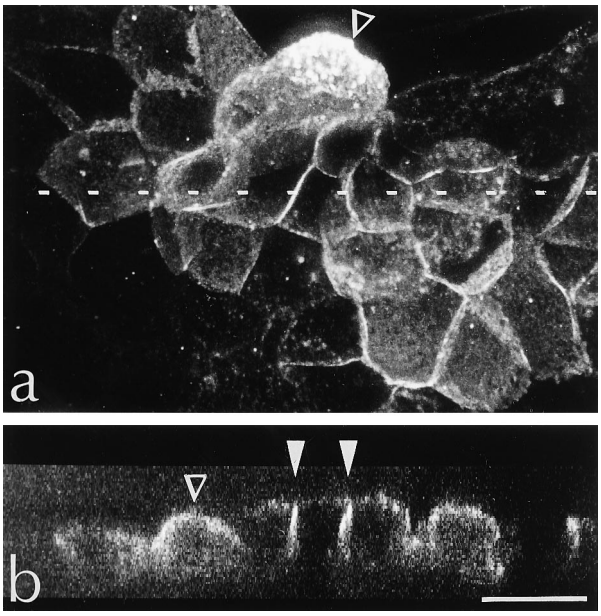

FIG. 3. Confocal laser-scanning micrograph of HCT-8/S cells fixed and immunostained with MAb CD6 plus FITC-conjugated goat anti-mouse IgG after 1 h of coculture with *E. histolytica* trophozoites. (a) Horizontal projection made from 26 sections tangential to the substrate. (b) Vertical projection made from five transverse sections. Closed triangles mark sites of accumulation of the immunosignal at lateral cell surfaces; open triangles indicate trophozoites. The dashed line marks the trace of transverse sectioning. Bar = 20 μm.

in over 40 experiments with enterocyte monolayers as well as with breast MCF-7/6 cells (Fig. 2e) and kidney MDCK cells (not illustrated). In contrast, the fibroblast cell types, ICIG-7 and MRC-5 (Fig. 2f), were immunonegative.

In the absence of trophozoites, we were unable to provoke CD6 epithelial immunostaining by mild trypsinization of the monolayer (up to the point of rounding of cells) or by treatment with neuraminidase or with 4-methylumbelliferyl-β-D-xyloside.

Contact-dependent transfer of an ameba-specific antigen to cell monolayers. The antigen detected on the monolayers by some ameba-specific antibodies seemed to be transferred by active trophozoites in a contact-dependent manner. Epithelial cell immunostaining by MAb CD6 did not occur under the following conditions: in cocultures held at 4°C; when fixed trophozoites were used to prepare cocultures; when monolayers were fixed prior to addition of live trophozoites; with 3-day- or 1-h-old trophozoite-conditioned medium or 1-h-old coculture-conditioned medium; or in cocultures in which direct contact between trophozoites and epithelial cells was prevented by an interposed tissue culture insert filter. Application of amebic sonicate or lysate to monolayers resulted in a random immunostaining pattern over the whole surface of the monolayer without accentuation at the lateral cell surface (Fig. 1D and E), confirming others' results (32).

Localization of ameba-specific antigen on the monolayer. Confocal microscopy indicated that the amebic antigen recognized by the CD6 MAb was located at the lateral surface of the epithelial cells (Fig. 3). Additional evidence for this localization was obtained by double immunostaining of the cocultures with CD6 and anti-E-cadherin MAbs. The immunosignals from both MAbs were colocalized, as shown in Fig. 4. While the E-cadherin immunosignal covered the whole monolayer, the CD6 signal remained restricted to distinct areas in the

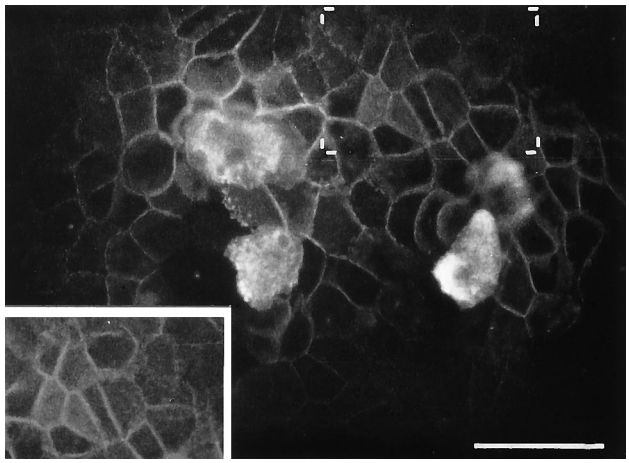

FIG. 4. Fluorescence micrographs of MCF7/6 cells fixed and doubly immunostained with CD6 and a rat monoclonal anti-E-cadherin antibody (inset from boxed area) after 1 h of coculture with *E. histolytica* trophozoites. The primary antibodies were detected with FITC-conjugated anti-mouse IgG (CD6) and biotinylated anti-rat IgG followed by streptavidin-Texas red (anti-E-cadherin). Bar = 50 μm.

vicinity of attached trophozoites. Limited access of the amebic antigen to its enterocyte binding site was suggested by two observations: the absence of epithelial immunostaining with fixed monolayers and a more prominent epithelial immunosignal with subconfluent monolayers (TER values of approximately 300 $\Omega \times cm^2$) than with confluent ones (TER values of about 1,000 $\Omega \times cm^2$). In cocultures with confluent MDCK cell monolayers, epithelial cell immunostaining was limited to a few spots. These observations are in line with the finding that undifferentiated Caco-2 cells were more accessible to trophozoites than postconfluent monolayers (17).

Relation of deposited antigen to monolayer destruction. Videomicrography of cocultures with enterocytic monolayers showed that enterocytes were released from the substrate in the vicinity of attached trophozoites after about 30 min, whereas enterocyte immunostaining was detected in analogous cultures after 5 min. Staining with fluorescein diacetate and propidium iodide demonstrated that some cells at the periphery of defects in the monolayer and in contact with amebae were dead (Fig. 5). This suggested that binding of *E. histolytica* CD6 antigen preceded and possibly signalled the initiation of epithelial cell cytolysis followed by release from the substratum, as suggested by others' experiments (28). Dead cells were not observed when sonicates were used instead of live trophozoites, as described previously by Ravdin (31).

Identification of the CD6 antigen. Surface binding of MAb CD6 to *E. histolytica* trophozoite lysates was demonstrated by immunofluorescent and immunogold staining (Fig. 6). On confocal sections of whole trophozoites, not permeabilized with Triton X-100 after fixation, a bright surface staining of all cells and vacuolar staining in many cells were visible (Fig. 6a). Immunogold-stained sections showed primarily surface staining (Fig. 6b). Surface capping by MAb CD6 on live trophozoites and the ability of this MAb to agglutinate fixed trophozoites were demonstrated (data not shown).

Fluorographs of SDS-PAGE-resolved, ^{35}S-labeled CD6 immunoprecipitates from trophozoite lysates revealed bands not seen in the control precipitates prepared without antibody. Three bands were around 170 kDa, and one band was near 35 kDa (Fig. 7, lane 2). This pattern closely resembled that obtained with immunoprecipitates prepared with MAb 7F-4 (Fig. 7, lane 3), which is directed against the 170-kDa Gal/GalNAc lectin subunit. The 170-kDa-like pattern was also present in immunoprecipitates prepared with the *E. histolytica* antisera 1553, 1554, and EH-002 (data not shown) that caused trophozoite-associated immunostaining of epithelial cells (Table 1). Under nonreducing conditions, both CD6 and 7F-4 MAbs immunoprecipitated a band of around 240 kDa (Fig. 7, lanes 5 and 6). Immunoprecipitates prepared with PL, an uncharacterized commercial MAb which did not show ameba-dependent immunostaining of epithelial cells (Fig. 1C), did not contain the 170-kDa band. Fluorographs of SDS-PAGE-resolved CD6 immunoprecipitates from ^{35}S-labeled trophozoite-free enterocytes did not show CD6-specific bands (data not shown).

Effects of sugars and of protease inhibitors on CD6 epithelial cell immunostaining. In untreated cocultures about 70% of the attached trophozoites were associated with epithelial im-

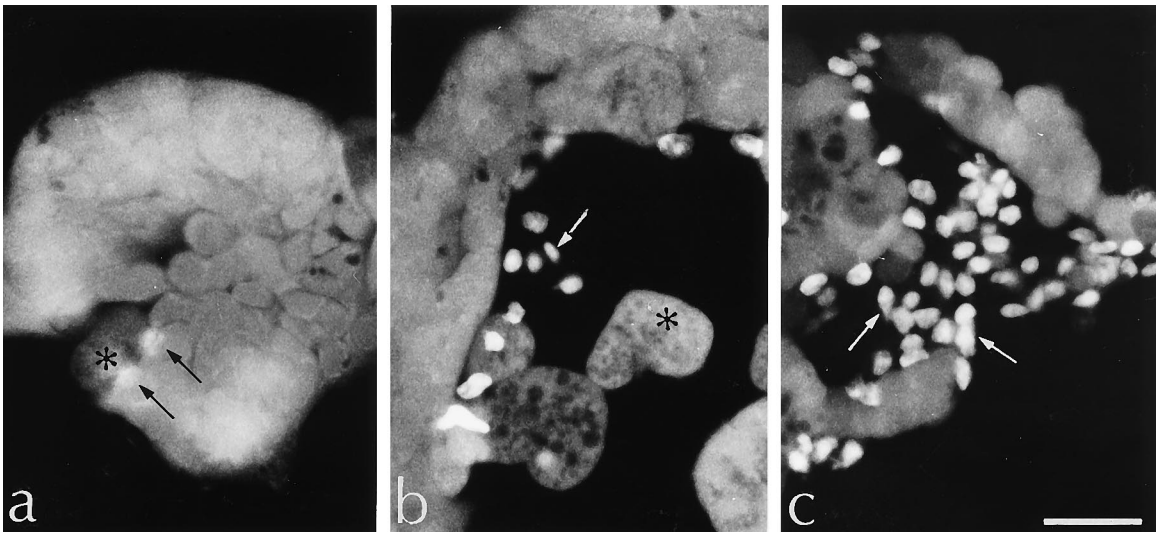

FIG. 5. Fluorescence micrographs made after vital staining with fluorescein diacetate and propidium iodide of trophozoites and HCT-8/S cells in coculture for 5 min (a), 15 min (b), and 60 min (c). The number of dead cells (arrows) increases with time of coculture. Asterisks mark some of the trophozoites disrupting the monolayer. Bar = 50 μm.

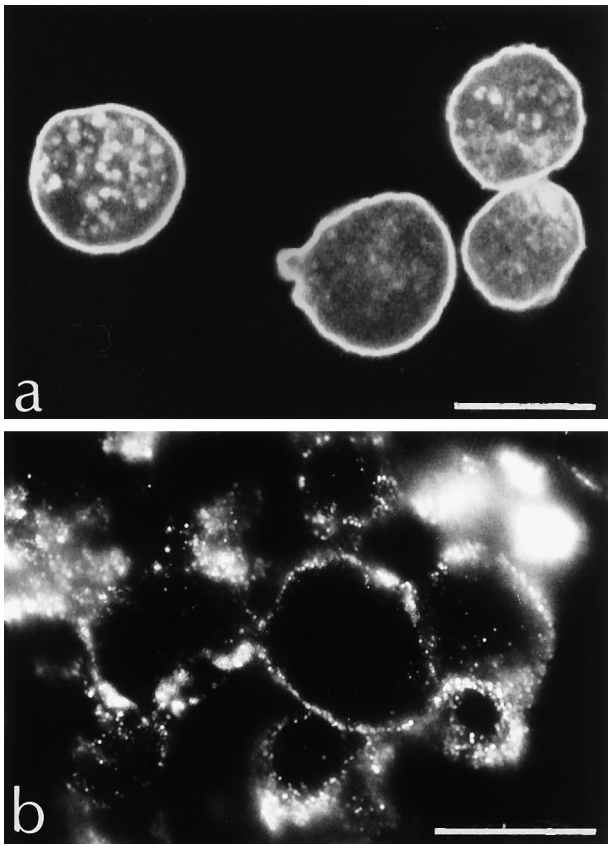

FIG. 6. Confocal fluorescence (a) and epipolarization (b) micrographs of trophozoites fixed with PF and immunostained with MAb CD6 revealed with FITC-conjugated anti-mouse IgG (a) or by immunogold-silver enhancement (b). One-micrometer-thick optical sections at the mid plane (a); 3-μm-thick histological sections (b). Bar = 25 μm.

munostaining in their vicinity. A reduction in the number of attached trophozoites to about 20% of the level in untreated cocultures was seen with D-(+)-galactose, β-lactose, or *N*-acetyl-D-galactosamine. Here also about 70% of the attached trophozoites were associated with epithelial immunostaining, indicating that the total amount of amebic antigen transferred is only 14% of that which occurs in controls without these sugars. *N*,*N*′,*N*″-Triacetylchitotriose, asialofetuin, and heparin had no effect on attachment or on trophozoite-associated epithelial immunostaining. Treatment of trophozoites before and during coculture with various protease inhibitors had no effect on epithelial immunostaining. However, with some protease inhibitors (*N*-ethylmaleimide at 0.2 to 2 mM, pHMB at 10 mM, IA at 0.5 and 5 mM, and PMA at 1 and 10 mM) trophozoites failed to attach, probably because of toxicity, and epithelial immunostaining was absent.

DISCUSSION

The principal finding of this study was that the ameba-specific mouse MAb CD6 immunostained the lateral surface of epithelial cells in the vicinity of attached trophozoites. Our explanation is that the antigen recognized by the CD6 MAb, or a portion of it containing the CD6 epitope, was transferred from the trophozoite to the epithelial cell surface during the period of coculture.

It is less likely that trophozoites uncovered an epithelial epitope recognized by the CD6 MAb. Such an uncovering activity of the trophozoites could not be mimicked by agents known to remove cell surface residues that could be responsible for hindering access of the antibody. Accordingly, an attempt to block the presumed uncovering activity by a variety of protease inhibitors failed. If epitope uncovering was the mechanism, lysates presumably containing the specific proteases would have accomplished epithelial immunostaining. Moreover, CD6 did not immunoprecipitate specific bands from total lysates of radiolabeled enterocytes.

Fluorographs of SDS-PAGE-resolved immunoprecipitates obtained with MAb CD6 compared with those of immunoprecipitates obtained with MAb 7F-4, under reducing and nonreducing conditions, provided evidence that the CD6 MAb is directed against the Gal/GalNAc-specific lectin. The presence of two to three bands of around 170 kDa may be ascribed to differences in glycosylation of the lectin large subunit (34) or to coimmunoprecipitation of unidentified proteins. The Gal/Gal NAc-specific lectin, described and characterized by others, is presumed to interact with the target cell surface during lectin-glycan binding (28, 29, 34–36, 43).

Our results indicate that antigen transfer was a biological phenomenon manifesting specificity and depending on the action of live cells. CD6 immunostaining occurred in cocultures with all epithelial cell lines tested but not with fibroblasts, indicating some degree of target cell specificity. CD6 binding was limited to the epithelial cell surface and appeared to be most prominent on the lateral surface in regions of epithelial cell-cell contact, indicating transfer site specificity. We presume that the putative epithelial binding site is situated basally to tight junctions. In double immunostainings, the amebic antigen colocalized with E-cadherin, a marker of adherence junctions. Like immunostaining of this marker, CD6 immunostaining in the vicinity of trophozoites necessitated Triton X-100 permeabilization. Amebic antigen transfer to epithelial cells was not observed if either the trophozoites or the monolayer was fixed before admixture, indicating a requirement for live donor and recipient cells. While Gal/GalNAc-inhibitable at-

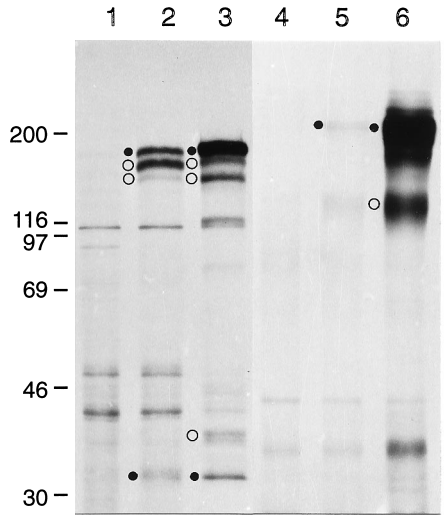

FIG. 7. Fluorograph of an SDS–7.5% PAGE gel under reducing (lanes 1 to 3) and nonreducing (lanes 4 to 6) conditions containing immunoprecipitates from trophozoites metabolically labeled with Tran^{35}S-Label, lysed, and immunoprecipitated without antibody (lanes 1 and 4) or with MAb CD6 (lanes 2 and 5) or 7F-4 (lanes 3 and 6). The positions of molecular mass markers (in kilodaltons) are shown on the left. Closed circles indicate the positions of the nonreduced Gal-specific lectin, its 170-kDa heavy subunit, and its 35-kDa light subunit; open circles mark unidentified but specific bands.

tachment of trophozoites to target cells occurred at 4°C (29, 33, 35), transfer of the amebic CD6-positive antigen to epithelial cells did not. The effect of Gal-containing compounds illustrated the need for attachment of trophozoites to the enterocytes in order to produce enterocyte immunostaining at 37°C. Attachment of trophozoites (about 20% of the level in untreated cocultures) in the presence of D-(+)-galactose, β-lactose, or *N*-acetyl-D-galactosamine may point to an incomplete effect of the sugars or to attachment via another molecule. This incomplete inhibition may also hold for transfer of the amebic antigen, or this antigen might bind via a nonlectin domain. Indeed, lectins such as the slime mold lectin discoidin I and the vertebrate elastin receptor may have different domains. The binding functions of some domains were insensitive to lectin-specific sugar residues (8), unlike the adherence and cytotoxicity of *E. histolytica* trophozoites (3, 4, 16, 19, 21, 27, 33). The binding domain on the amebic antigen did not correspond to the domain covered by the CD6 MAb or the 1553 and 1554 polyclonal antibodies. Prebinding of these antibodies to trophozoites before coculture did not inhibit epithelial immunostaining (our unpublished results).

It is unlikely that antigen free in the medium could account for the observed immunostaining patterns. The CD6-positive antigen was detected by immunofluorescence in trophozoite sonicates and detergent lysates. However, only a random immunostaining of the exposed surface of monolayers, without any tendency toward accumulation at the lateral epithelial cell surface, was observed after incubation with these cell-free preparations. Immunostaining by CD6 was not observed when ameba-conditioned medium prepared in several ways was incubated with monolayers or when trophozoite-target cell contact was prevented by tissue culture insert filters. We concluded from these data that a contact-dependent mechanism requiring metabolically active trophozoites leads to the deposition of the ameba-specific surface molecule at the epithelial target cell membrane. The finding of epithelial immunopositivity at some distance from attached trophozoites and the increase of immunopositivity with time of coculture could be explained by a continuous contact-dependent transfer of antigens from trophozoites performing on spot motility (15).

Antibodies to several other amebic proteins failed to bind to target cells, indicating specificity in terms of the molecule transferred. The fact that epithelial immunostaining in the vicinity of attached trophozoites was observed also with our polyclonal antibodies 1553 and 1554 pleads in favor of transfer of the Gal/GalNAc-specific lectin or its 170-kDa subunit, known to be immunodominant. Why the antibodies recognizing the cysteine-rich extracellular domain of the Gal/GalNAc-specific 170-kDa lectin (28) failed to stain the epithelial cells may be explained in several ways. After transfer, some epitopes may be inaccessible through the way of binding of the antigen to the enterocyte. Alternatively, the structure of the amebic antigen may have been altered in a way that rendered the epitopes on the cysteine-rich domain unrecognizable by antibodies that do bind to the parental Gal-specific lectin (28). Furthermore, it is possible that only a part of the Gal-specific lectin is transferred. These possibilities are in line with the results of steric competition experiments. CD6 but not antibodies against the cysteine-rich domain of the 170-kDa lectin subunit hindered binding of biotinylated CD6 antibodies (our unpublished results).

One can only speculate concerning the relevance of amebic antigen transfer for the mechanism of *E. histolytica* cytopathogenicity. Other investigators have reported a decrease in TER as an early event following trophozoite attachment to Caco-2 monolayers, suggesting weakening of the tight junctions of the monolayer (17). We observed a negative correlation of the binding ability of MAb CD6 with TER. This suggests that access of trophozoites to the lateral surfaces of the target cells was required for amebic antigen transfer. One possibility is that transfer starts at small preexisting defects and that it is rapidly amplified as soon as trophozoites reach the basolateral surface of the epithelial cells. Alternatively, since tight junctions are very sensitive to subtle environmental changes, trophozoites might well have been responsible for the initial opening, as proposed by others (17, 18, 20). Amebic antigen transfer was detected as early as 5 min following seeding of trophozoites, whereas target cell death was not detected sooner than 15 min after coculture. Thus, antigen transfer could play a role also in the breakdown of target cell membrane integrity. Molecular transfer from the aggressor to the target cell has been reported for *E. histolytica* (14), for other parasites (38, 40), and for cancer invasion (15, 25, 41).

It has been well established that the activity of the Gal-specific lectin is contact dependent, requires entire amebae, and cannot be mimicked by sonicates or conditioned medium (31, 32). Our observations confirm these findings and add a new aspect, namely, early translocation of the lectin to target cells. We think that because of the novelty of this phenomenon and the potential that it will be relevant to the mechanism of *E. histolytica* cytopathogenicity, further investigation is warranted.

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