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A New In Vitro Model of *Entamoeba histolytica* Adhesion, Using the Human Colon Carcinoma Cell Line Caco-2: Scanning Electron Microscopic Study

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The human colon carcinoma cell line Caco-2, which is widely used to study the adhesion and cytotoxicity of enterobacteria, was used to investigate the adhesion of the trophozoites of *Entamoeba histolytica*. We observed a high percentage of adhesion of amoebae to Caco-2 cells. Scanning electron microscopy showed that amoebial membrane structures were involved in adhesion and the cytolytic action. These differentiated cells should prove to be a useful model system for investigation of the pathogenic action of amoebae.

Three consecutive processes are involved in the invasion of tissues by the pathogen *Entamoeba histolytica*: adhesion of the amoebae to target cells, contact cytolytic action, and phagocytosis. Adhesion is a crucial step in the expression of the cytopathogenicity of this amoeba (16, 21). Study of the cytopathogenic processes involved in human infections with *E. histolytica* would be aided by the development of a suitable in vitro model (11, 22). Caco-2 cells, derived from a human colon adenocarcinoma (6, 23), are now widely used to study the adhesion, invasion, and cytotoxicity of pathogenic bacteria (3, 7, 8, 21).

In this study, to investigate the cytopathogenic action of amoebae on Caco-2 cells in culture, we determined the percent adhesion, and we used scanning electron microscopy (SEM) to examine the adhesion process and cytolytic action in more detail. Surface features of the trophozoites, such as endocytic vesicles, pseudopods, and filipods, which are involved in adhesion (13) were observed. The cytolytic action of the amoebae was evidenced from the appearance of zones of lysis after contact of the amoebae with the epithelial monolayer.

MATERIALS AND METHODS

The Caco-2 cells, established by Fogh (Sloan-Kettering Memorial Cancer Center, New York) in 1974 (6), were routinely cultured on coverslips (glass support, 22 by 22 mm, degreased and sterilized) in six-well dishes (Corning; reference no. 25810) and covered with Dulbecco's modified Eagle's minimal medium (DMEM). Before use, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1 μg/ml), 20% decomplemented fetal calf serum (15 min, 60°C), and 1% nonessential amino acids were added. The cultures were incubated at 37°C in a 90% air–10% CO_2 mixture (ESPEC; Flobio). The culture medium was renewed daily.

The Caco-2 cells used in the different experiments (adhesion, cytotoxicity, and SEM) were cultured under the same conditions. To obtain a monolayer of target cells which completely covered the coverslip, the cells were used on day 15 of culture. Each coverslip contained about 1.3×10^6 confluent cells, and at this stage of growth, they express most fully the differentiation features of enterocytes (23).

E. histolytica clone A (E. Orozco, National Polytechnic Institute, Experimental Pathology, Mexico City, Mexico) was isolated in semisolid agar medium from the HM1 axenic strain (10). It contains a homogeneous population of virulent cytopathogenic trophozoites with strong adherent properties and high phagocytic activity (20, 29). Clone A was cultured axenically at 36°C on BIS 33 medium (4) enriched with 15% bovine serum (Hyclone; reference no. 41 112) and 3% of a mixture of vitamins and Tween 80 (reference no. 52 07277; Hazleton, Glasgow, U.K.). Trophozoites in the exponential growth phase (72-h culture) were removed by cooling for 5 min at 4°C. After centrifugation (Sorval R.L. 5B; Dupont Instruments) at 200 × *g* for 5 min at 4°C, the pellet was washed three times with BIS 33 without serum to avoid disturbing amoeba-target cell binding (1, 28). After resuspension in BIS 33 without serum, the trophozoite concentration was measured in a hemacytometer. Although the integrity and viability of the trophozoites, which are required for expression of their adhesive and cytopathogenic properties, are normally preserved under these conditions (16, 19, 26), viability was checked before each experiment by phase-contrast microscopy (refringent and mobile cells). We observed no loss of viability after incubation at 37°C for 15 min to 2 h.

For the adhesion test, each coverslip with a target cell monolayer was washed twice with sterile phosphate-buffered saline (PBS) and then covered with 1 ml of DMEM immediately before adding the suspension of amoebae (3×10^5 trophozoites per well; one amoeba per four Caco-2 cells). We checked that the Caco-2 cells were not affected by the presence of the *E. histolytica* medium (BIS 33), as has been observed for the culture medium of bacteria (3) and *Cryptosporidium parvum* (5). The Caco-2 cell-amoeba mix was incubated for 15 min at 37°C, and the medium was removed and kept for estimation of the number of nonadhering amoebae. The coverslips were washed three times with PBS by swirling gently to remove nonadherent amoebae. The number of adherent trophozoites, expressed as percent adhesion, was taken as the difference between the initial number of amoebae added (3×10^5/ml) and the total number of amoebae in the medium removed and in the wash liquid.

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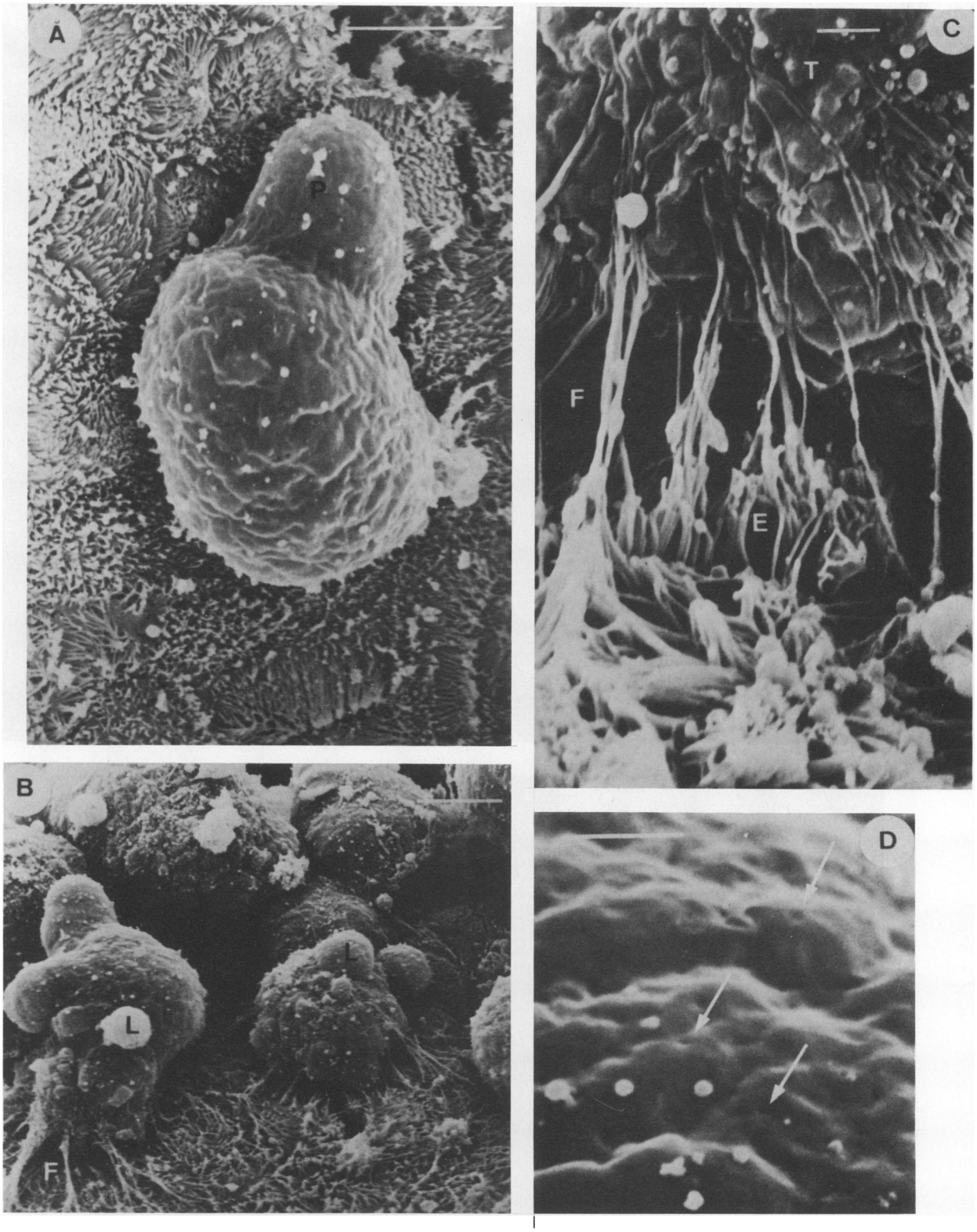

FIG. 1. (A) Trophozoite on the surface of differentiated Caco-2 cells with or without deformation of the brush border. Note pseudopods (P). Bar, 10 μm. (B) Adhesion of trophozoites to enterocytes via filipods (F). Note some small pseudopods of spherical appearance with a smooth surface (L). Bar, 10 μm. (C) Binding of trophozoite (T) to an enterocyte. Note filipods (F) pulling brush border (E) towards trophozoite. Bar, 1 μm. (D) Free surface of trophozoite. Note creased appearance with numerous pinocytic orifices (arrows). Bar, 10 μm.

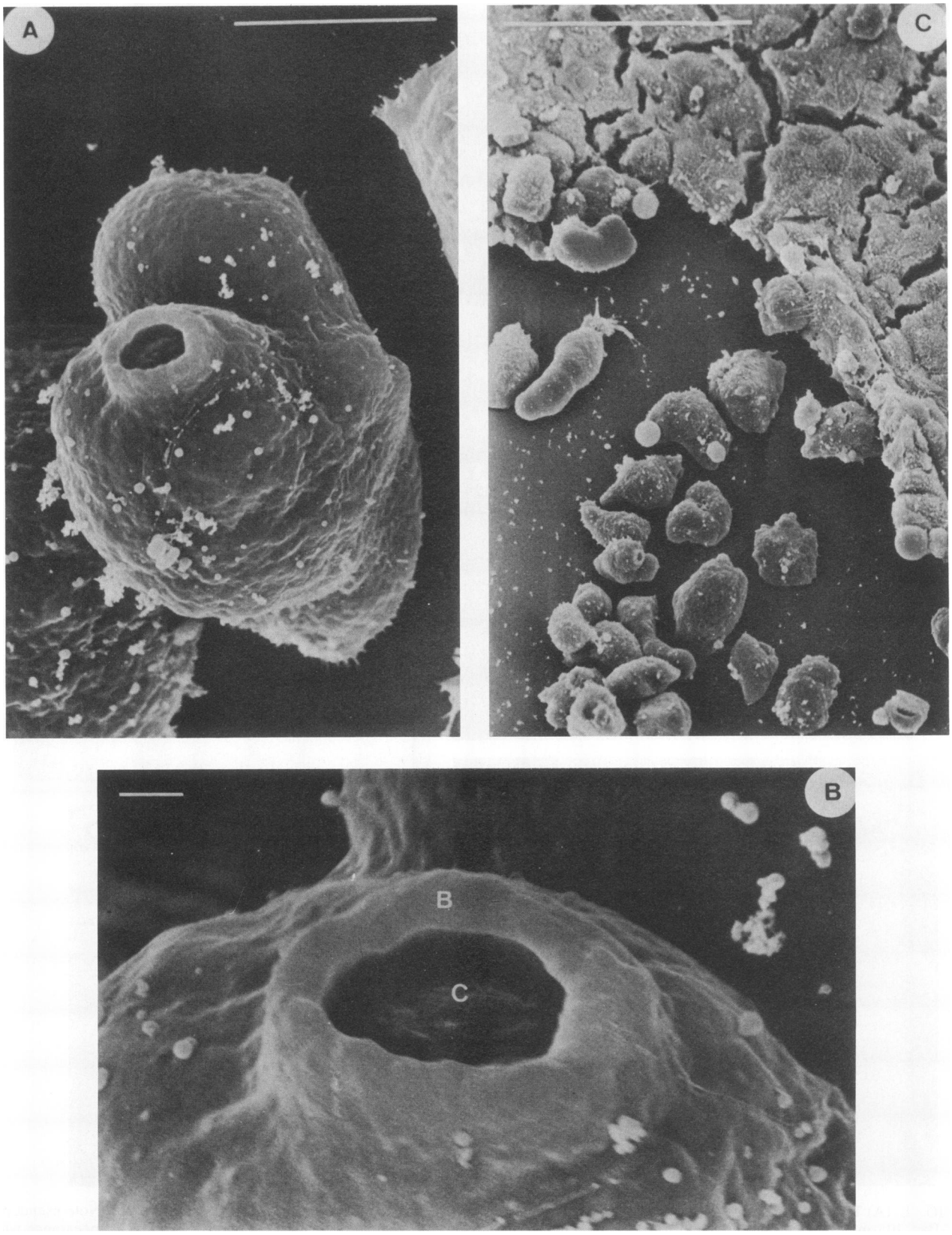

FIG. 2. (A) Appearance of trophozoite after cytolysis of intestinal cells in culture. Note phagocytic vacuole. Bar, 10 μm. (B) Detail of phagocytic vacuole. Note thickening (B) around the phagocytic channel (C) formed by the plasma membrane. Bar, 1 μm. (C) Phagocytic patch in a monocellular epithelial layer from cytolytic action of *E. histolytica*. Bar, 100 μm.

Each experiment was carried out in duplicate on three different days, and each count was done twice, so the total number of adherent trophozoites for each experiment is given as the mean of four cell counts. In the supernatant, all counts were carried out in duplicate (Table 1).

The cytotoxic effect of the amoebae was investigated by the same procedure, except that the duration of incubation of amoebae and cells was increased from 15 min to 2 h.

For the SEM study, the coverslips with confluent or lysed cells were washed three times with phosphate-buffered saline, fixed in 2% glutaraldehyde (1 h at room temperature), postfixed in 2% osmic acid (30 min in the dark), dehydrated in a series of graded alcohol baths, and then subjected to critical-point drying in CO_2 (maximum temperature, 34°C; maximum pressure, 73 atm; Balzers CPD 010). The Caco-2 cells and the amoebae were coated with gold (Sputter Coater S 150) before examination in a Jeol JSM 840 A scanning electron microscope at 12 kV.

RESULTS

The adhesion tests performed with the Caco-2 intestinal cells showed that 72% ± 2.73% of the amoebae in the suspension adhered to the target cells (Table 1).

The SEM examination showed that the amoebae adhered at the brush border, a characteristic feature of differentiated human intestinal cells (Fig. 1A). Various surface structures which are thought to be involved in adhesion were observed. Filipods (Fig. 1B and C) with lengths up to 10 μm and diameters below 0.3 μm were seen to sprout from the pole of amoebae in contact with the target cell. They radiated from this zone, occupying an area on the cell that was greater than that masked by the amoebae. The free surface of the trophozoites appeared irregular (Fig. 1A and D), presenting orifices, which were thought to be pinocytic vesicles (Fig. 1B), and pseudopods, spherical or elongated protrusions with diameters ranging from 1 to 8 μm (Fig. 1A and B). Phagocytic vesicles were less frequently observed. They were probably localized in the contact zone, as they were abundant after cytolysis, when the amoebae became detached from the Caco-2 cells (Fig. 2A and B). The patchy disappearance of the epithelial layer was assumed to be due to cytolysis (Fig. 2C).

TABLE 1. Adhesion of *E. histolytica* trophozoites to Caco-2 cells[a]

10^3 Trophozoites added and count no.	10^3 Trophozoites in medium and wash liquid		Calculated % adhesion		Avg % adhesion
	Assay 1	Assay 2	Assay 1	Assay 2	
325 ± 16					71 ± 2.9
First count	91	84	72	74	
Second count	107	94	67	71	
334 ± 17					74.75 ± 2.20
First count	92	80	72	76	
Second count	86	76	74	77	
306 ± 20					70.25 ± 3.25
First count	87	80	72	74	
Second count	100	97	67	68	

[a] For each of three assays on different dates, the number of trophozoites added to Caco-2 cells was determined (mean ± standard error of the mean, $n = 4$). On each date, two separate assays were done, each in duplicate (assays 1 and 2, first and second counts). The percent adhesion was calculated as described in the text. The overall average percent adhesion for all assays was 72% ± 2.73%.

DISCUSSION

The experimental conditions (temperature, contact time, ratio of amoebae to epithelial cells) were similar to those used for other target cell systems, such as human erythrocytes (2), BHK and CHO cells in culture (13, 20), and human intestinal cells (14). The percent adhesion was close to that reported by Kobiler and Mirelman (14) with human intestinal cells in culture (Henlé 407). The estimated percent adherence to the Caco-2 cells is given here to enable comparison with the results of other systems. The percent adhesion, calculated from the difference between the total number of amoebae placed in contact with the target cells and the number counted in the supernatant and wash liquid, has an inevitable margin of error; amoebae may adhere to the bottom of each well around the coverslip (about 20% of the total surface area of the coverslip). Our primary aim was to evaluate this system for study of the different components in the pathogenicity of this amoeba (adhesion and cytolysis) rather than to determine their exact degree of adherence.

The human intestinal Caco-2 cell line thus appears to be a good model system for investigation of the adhesion of amoebae to human cells and the ensuing cytolysis. With respect to previously used cell systems, such as animal cell lines (12, 26), and human (14) or animal (26) enterocytes, Caco-2 cells are physiologically closer to the natural targets of the amoebae, as, in culture, they exhibit the differentiated features of enterocytes (23), such as cell polarity, apical brush border, and expression of intestinal hydrolases (32).

The new model system represented by the adhesion of amoebae to Caco-2 cells also involves membrane structures observed in other systems (12, 13, 17, 31) and displays the characteristic stages in the cytopathogenic process (25), namely, contact between amoebae and target cells leading to cytolysis, which are observed in both human (24) and animal (18) amebiasis.

This human intestinal cell line could be of value for study of the interaction between amoebial adhesins (29, 30) and specific receptors on target cells.

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REFERENCES

1. **Bos, H. J., and A. A. Van Den Eijk.** 1980. Serum inhibited toxicity of *Entamoeba histolytica*. Arch. Invest. Med. (Mexico) **11**:135–141.
2. **Cano-Mancera, R., and R. Lopez-Revilla.** 1987. Inhibition of the adhesion of *Entamoeba histolytica* trophozoites to human erythrocytes by carbohydrates. Parasitol. Res. **74**:18–22.
3. **Darfeuille-Michaud, A., D. Aubel, G. Chauviere, C. Rich, M. Bourges, A. Servin, and B. Joly.** 1990. Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. Infect. Immun. **58**:893–902.
4. **Diamond, L. S., D. 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.
5. **Flanigan, T., A. I. Toshika, R. Marshall, R. Soave, A. Masamichi, and C. Kaetzel.** 1991. A sexual development of *Cryptosporidium parvum* within a differentiated human enterocyte cell line. Infect. Immun. **59**:234–239.
6. **Fogh, J., J. M. Fogh, and T. Orfeo.** 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. **59**:221–226.
7. **Gahring, L., F. Heffron, B. Brettfinlay, and S. Falkow.** 1990. Invasion and replication of *Salmonella typhimurium* in animal

cells. Infect. Immun. **58**:443–448.
8. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocytelike cell line Caco-2. Infect. Immun. **55**:2822–2829.
9. **Garcia-Riviera, G., T. Sanchez, E. Orozco, and G. Guarneros.** 1982. Aislamiento de clonas de *E. histolytica* deficientes en adhesion a eritrocitos humanos. Arch. Invest. Med. (Mexico) **13**:129–136.
10. **Gillin, F. D., and L. S. Diamond.** 1978. Clonal growth of *Entamoeba histolytica* in agar. J. Protozool. **25**:539–543.
11. **Gitler, C., E. Calef, and I. Rosenberg.** 1984. Cytopathogenicity of *Entamoeba histolytica*. Phil. Trans. R. Soc. London **307**:73–85.
12. **Gonzalez-Robles, A., and A. Martinez-Palomo.** 1982. Morfologia de trofozoitos de *Entamoeba histolytica* en substratos naturales. Estudio mediante microscopia electronica de barrido. Arch. Invest. Med. (Mexico) **13**:145–157.
13. **Gonzalez-Robles, A., and A. Martinez-Palomo.** 1983. Scanning electron microscopy of attached trophozoites of pathogenic *Entamoeba histolytica*. J. Protozool. **30**:692–700.
14. **Kobiler, D., and D. Mirelman.** 1981. Adhesion of *Entamoeba histolytica* trophozoites to monolayers of human cells. J. Infect. Dis. **144**:539–546.
15. **Leitch, G. J., A. V. Dickey, I. A. Udezulu, and B. Gordon.** 1985. *Entamoeba histolytica* trophozoites in the lumen and mucus blanquet of rat colons studied in vivo. Infect. Immun. **47**:68–73.
16. **Lopez-Revilla, R., and R. Cano-Mancera.** 1982. Adhesion of *Entamoeba histolytica* trophozoites to human erythrocytes. Infect. Immun. **37**:881–885.
17. **Lusbaugh, W. B., and F. E. Pittman.** 1979. Microscopic observations on filopodia of *Entamoeba histolytica*. J. Protozool. **26**:186–195.
18. **Martinez-Palomo, A., A. Gonzales-Robles, B. Chabez, E. Orozco, S. Fernandez-Castelo, and A. Cervantes.** 1985. Structural bases of the cytolytic mechanisms of *Entamoeba histolytica*. J. Protozool. **32**:166–175.
19. **Orozco, E., S. Fernandez-Castelo, and A. Martinez-Palomo.** 1982. Deteccion del dano cellular temprano producido por *Entamoeba histolytica* sobre epitelios. Arch. Invest. Med. (Mexico) **13**:164–175.
20. **Orozco, E., A. Martinez-Palomo, A. Gonzales-Robles, G. Guarneros, and J. Mora-Galindo.** 1982. Las interacciones entre lectina y receptor median la adherencia de *Entamoeba histolytica* a celulas epiteliales. Relacion de la adhesion con la virulencia de las cepas. Arch. Invest. Med. (Mexico) **13**:159–166.
21. **Panigrami, P., B. D. Tall, R. Russel, L. Detolla, and J. Glennmorris.** 1990. Development of an in vitro model for study of non-O1 *Vibrio cholerae* virulence using Caco-2 cells. Infect. Immun. **58**:3415–3424.
22. **Petri, W. A., and J. I. Ravdin.** 1988. *In vitro* models of amebic pathogenesis, p. 191–204. *In* J. Ravdin (ed.). Amebiasis: human infection by *Entamoeba histolytica*. John Wiley & Sons, Inc., New York.
23. **Pinto, M., S. Appay, P. Simon-Assmann, G. Chevalier, N. Dracopoli, J. Fogh, and A. Zweibaum.** 1982. Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. Biol. Cell. **44**:193–196.
24. **Pittman, F. E., W. Elhashimi, and J. C. Pittman.** 1973. Studies of human amebiasis. II. Light and electron microscopic observations of colonic mucosa and exudate in acute amebia colitis. Gastroenterology **65**:588–603.
25. **Ravdin, J. I., B. Croft, and R. L. Guerrant.** 1980. Cytopathogenic mechanisms of *Entamoeba histolytica*. J. Exp. Med. **152**:377–390.
26. **Ravdin, J. I., and R. L. Guerrant.** 1981. Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*: study with mammalian tissue culture cells and human erythrocytes. J. Clin. Invest. **68**:1305–1313.
27. **Ravdin, J. I., J. E. John, L. 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.
28. **Reed, S. L., P. G. Sargeaunt, and A. I. Biaude.** 1983. Resistance to lysis by human serum of pathogenic *Entamoeba histolytica*. Trans. R. Soc. Trop. Med. Hyg. **77**:248–253.
29. **Rodriguez, M. A., F. Hernandez, L. Santos, A. Valdez, and E. Orozco.** 1989. *Entamoeba histolytica*: molecules involved in the target cell-parasite relationship. Mol. Biochem. Parasitol. **37**: 87–100.
30. **Rosales-Encina, J. L., I. Meza, A. Lopez-de-Leon, P. Talamas-Rohana, and M. Roskind.** 1987. Isolation of 220-kilodalton protein with lectin properties from a virulent strain of *Entamoeba histolytica*. J. Infect. Dis. **156**:790–797.
31. **Sepulveda, B.** 1982. Progress in amebiasis. Scand. J. Gastroenterol. **17**:153–163.
32. **Zweibaum, A., M. Laburthe, E. Grasset, and D. Louvard.** 1990. Use of cultured cell lines in studies of intestinal cell differentiation and function. Handbook of Physiology, in press.