Entamoeba histolytica Trophozoites in the Lumen and Mucus Blanket of Rat Colons Studied In Vivo

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Trophozoites of *Entamoeba histolytica* HM-1 were cultivated axenically in TYI-S medium. The amoebae were then transferred into this medium lacking serum (TYI) and inoculated into in vivo colon loops of adult Sprague-Dawley rats. The trophozoites were rapidly absorbed by the mucus, and few were found free in the luminal fluid by 1 h. By 4 h, the amoebae began to reappear in the lumen, aggregated in sloughed mucus blanket fragments. The colon was examined histologically and by scanning electron microscopy. There was no evidence of invasion or even brush-border attachment by the trophozoites within 4 h. In TYI, trophozoite motility was low. Exposure to the colonic lumen environment for 5 min in this medium significantly increased motility. However, as the trophozoites became absorbed to mucus fragments, their observed motility virtually ceased despite some morphological evidence of pseudopod extension. Erythrophagocytosis was not significantly affected by either exposing trophozoites to TYI washings of the colonic lumen, or by the more complete medium, TYI-S, in which the amoebae were significantly more motile. Two major mucus glycoprotein oligosaccharide end-group sugars, L-fucose and N-acetyl-neuraminic acid, were tested for their effects on trophozoite motility in both TYI and TYI-S. L-Fucose reduced motility; the sialic acid increased motility. It is concluded that the intestinal lumen contains several compartments, including the luminal fluid and the mucus blanket, and that *Entamoeba* trophozoites exist in a highly motile state in the former and a low motility state in the latter. The mucus blanket provided a significant barrier to trophozoite access to intestinal epithelium target tissue.

Trophozoites of the pathogenic amoeba *Entamoeba histolytica* are able to breach the mucosal barrier of the large intestine to cause erosive lesions in the intestine (2). If the infection continues, extraintestinal lesions may also develop, particularly in the liver (17). The initial steps in the trophozoite invasion of the intestinal mucosa may involve (i) association of the trophozoites with intestinal epithelial cells via some amoeba surface lectins (6, 24); (ii) some contact killing of the attached epithelial cells (5, 19); (iii) disruption of the epithelium tight junctions (15); and (iv) movement of the trophozoites between the epithelial cells (22). This last step may require prior disruption of the epithelium tight junctions, or it may be accomplished mechanically by the trophozoites simply pushing their way between the epithelial cells (21). Phagocytosis of the mucosal cells probably occurs after the steps described above.

A variety of cytotoxins, cytopathic factors, and enterotoxins have been identified in *E. histolytica* trophozoites (1, 7, 9, 11, 18, 25). The presence or absence of these various factors in particular amoeba strains may or may not be an accurate predictor of strain pathogenicity (8, 10). The phagocytic activity of a particular strain has also been suggested to correlate with its pathogenicity (23). Host variables have further confounded studies of *E. histolytica* invasion of the intestine. Although it is relatively simple to demonstrate contact killing and phagocytosis of isolated cells, individually or in confluent monolayers (7, 15, 18), it is more difficult to demonstrate invasion of the intact mucosa. Germfree guinea pig intestine can be invaded by *Entamoeba* trophozoites injected intracecally (22), and some rat strains may be more susceptible to *E. histolytica* trophozoite intestinal invasion than others (13).

In our hands, HM-1 *Entamoeba* trophozoites readily phagocytized colonic enterocytes obtained from adult Sprague-Dawley rats, but they were unable to produce intestinal amebiasis after intracecal inoculation. This suggests that luminal conditions or the mucus blanket prevented trophozoite invasion of the mucosa. The in vivo colonic loop of the adult Sprague-Dawley rat was therefore chosen as a model for studying the behavior of *Entamoeba* trophozoites in the commensal phase, with a view to gaining an understanding of the role of the mucus blanket in preventing tissue invasion.

MATERIALS AND METHODS

Trophozoites. Trophozoites of the pathogenic strain of *E. histolytica*, HM-1, were cultivated axenically in TYI-S (3). The amoebae were harvested in late growth phase before use. Trophozoites were inoculated into in vivo rat colon loops in TYI-S or TYI (the stock medium lacking the serum, but with osmotic pressure and pH adjusted to that of the TYI-S) after cultivation in TYI-S.

Rat colon model. Adult Sprague-Dawley rats of either sex were anesthetized with sodium pentobarbital. Procaine was used at the site of a laparotomy as a local anesthetic. A catheter was inserted ca. 10 cm distal to the cecum-colon junction. The colonic contents were backflushed into the cecum with saline, and a ligature was secured near the junction to form a proximal colon loop of 6 cm. The loop was then filled with 0.5 ml of TYI. At the end of 20 min, the luminal fluid was recovered and its volume was measured. Net secretion occurred in ca. 50% of the experimental animals, and no net water flux or absorption occurred in the remainder. The proximity of the proximal ligature to the cecum, and the TYI osmolarity (325 to 350 mOsm), proved

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to be among the factors determining whether absorption or secretion occurred in the loop.

Next the loop was inoculated with 0.5 ml of TYI-containing HM-1 *E. histolytica* trophozoites (10^6 per ml). At 5 min, 20 min, 1 h, or 4 h, the luminal fluid was removed, the volume was measured, and the trophozoites were counted with a hemacytometer. Loop tissue was removed, gently rinsed once in saline, and fixed either in neutral Formalin for subsequent histology or in 2.5% glutaraldehyde buffered to pH 7.4 with 150 mM cacodylate buffer for subsequent observation by scanning electron microscopy. Tissue was also frozen in acetone precooled with liquid N_2. Freeze substitution was then performed by storing this tissue in acetone at $-60°C$ for 30 days with three changes of solvent. Both the fixed and freeze-substituted tissues were dried by critical point drying and, after sputter coating with gold-paladium, they were examined with an ETEC model OS 121 microscope (ETEC Corp., Hayward, Calif.).

In some experiments, the colonic mucus blanket was harvested at the end of the experimental period. This was performed after removal of the luminal fluid by first filling the loop lumen with 1 ml of 1-mg/ml cetylpyridinium bromide in saline (16). Five minutes later, this solution was removed and pooled with three subsequent saline washes. The precipitated mucus blanket, harvested in this manner, was then examined by light microscopy. *Entamoeba* trophozoites remained viable in this cetylpridinium bromide-

saline solution for over 15 min as judged by trypan-blue exclusion and cell motility.

Trophozoite motility measurements. Harvested trophozoites were washed and then equilibrated at 37°C in either TYI-S or TYI for 30 min before use. Motility measurements were performed in a 37°C warm room. The solutions containing the amoebae were placed on a prewarmed hemacytometer slide, and motility was scored after the amoebae were observed for 5 min. Three scoring procedures were used: a subjective score (0 to 4+), the number of 1/16-mm grid squares crossed by a given trophozoite in 5 min, and the number of 1/16-mm lines interesected by the same amoeba in the same time period. In each assay, 10 trophozoites were randomly chosen, their motility was measured, and the data were averaged.

In some motility experiments, a mucin preparation (type I, Sigma Chemical Co., St. Louis, Mo.) was added to TYI-S or TYI media. L-Fucose, *N*-acetyl-D-galactosamine, or *N*-acetyl-neuraminic acid was also added to either the TYI-S or TYI medium 30 min before trophozoite motility was measured.

To test the effects of a sojourn in the rat colon lumen on HM-1 trophozoite motility, in vivo rat colon loops were inoculated with amoebae (10^6 trophozoites per ml) in TYI or TYI-S. Five minutes later, the luminal fluid was removed and the motility of the amoebae was measured. In this case, the selection of trophozoites was not random. Two subpopu-
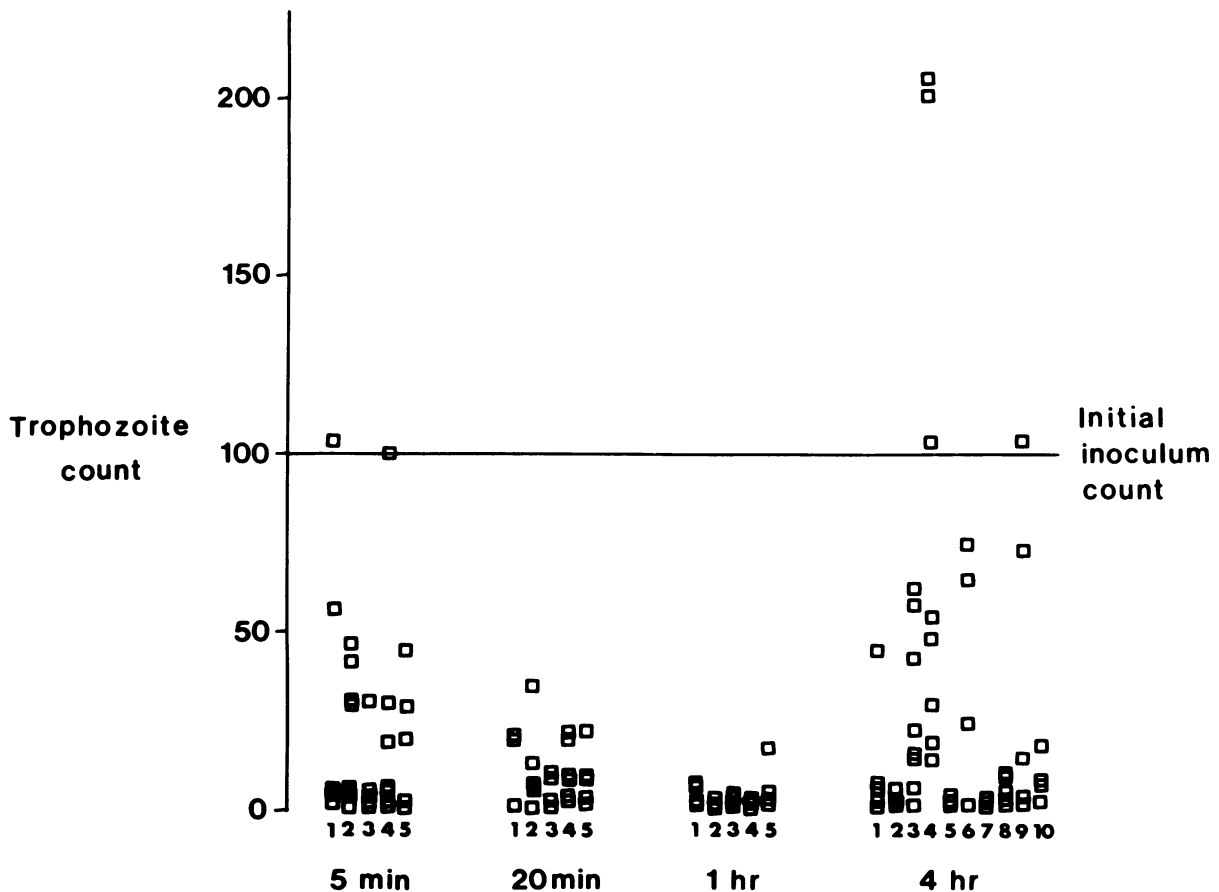

FIG. 1. *E. histolytica* trophozoites in TYI medium were inoculated into in vivo rat colon loops. The luminal fluid was removed, and the animals were sacrificed at 5 min, 20 min, 1 h, or 4 h. The number of trophozoites was counted in several luminal fluid samples from each of the numbered animals at each time period.

lations of trophozoites were distinguished in the luminal fluid, those that were free and those that were associated with mucus fragments. The motility of members of both subpopulations was measured.

Erythrocyte phagocytosis. Harvested trophozoites were washed and suspended in TYI medium at a concentration of 10^6 amoebae per ml, and 0.1 ml of this suspension was then added to 0.9 ml of TYI, TYI-S, or TYI which had been recovered 5 min after inoculation in in vivo rat colon loops. All of these solutions were adjusted to pH 6.3, the average pH of cultures at the time of harvest. After incubation at 37°C for 5 min, phagocytosis was initiated by the addition of 25 μl of a suspension of human erythrocytes in phosphate-buffered saline. After 10 min at 37°C, phagocytosis was terminated and free erythrocytes were lysed by the addition of 5 ml of cold water. After 1 min, the amoebae were fixed in 2.5% glutaraldehyde, and the number of amoebae exhibiting phagocytosis and the number of erythrocytes per amoeba were counted in the first 50 cells encountered on each slide.

RESULTS

Rat colon model. Trophozoites of *E. histolytica* HM-1 were inoculated into in vivo loops of rat colon. Figure 1 shows replicate counts of trophozoites in luminal fluid recovered 5 min, 20 min, 1 h, or 4 h after the trophozoite inoculation. By 5 min, the majority of trophozoite counts were less than the initial inoculum count, indicating a loss of amoebae from the recovered luminal fluid. This reduction in trophozoite counts continued into the 1-h samples. By 4 h after inoculation, trophozoites began to reappear in the recovered luminal fluid. These amoebae were generally attached in clumps to sloughed mucus fragments (Fig. 2c).

As there was no histological evidence that trophozoites invaded or even adsorbed to the colonic epithelium in 4 h, the data in Fig. 1 were interpreted as indicating that the amoebae were first adsorbed to the luminal surface of the mucus blanket, to be later sloughed off with mucus fragments. For confirmation of this interpretation, the colon mucus blanket was stripped off at 5 min, 20 min, 1 h, or 4 h after trophozoite inoculation, using cetylpyridinium bromide. Figure 2a shows this mucus blanket removed 5 min after trophozoite inoculation. Some areas of the recovered mucus were heavily infested with amoebae; other areas were clear. Mucus blanket recovered 4 h after trophozoite inoculation contained considerably fewer amoebae (Fig. 2b).

Much of the superficial mucus blanket was lost during fixation. Scanning electron microscopy of glutaraldehyde-fixed or freeze-substituted preparations of rat colon inoculated with 5×10^5 trophozoites failed to demonstrate many amoebae. Typically, by 1 h post-inoculation, only 2 or 3 trophozoites were visible on a mucosal surface of ca. 20 mm². The few amoebae that were visible in the scanning electron microscope preparations were small in size and usually covered in mucus, at the openings of crypts. Figure 3 shows a mucus-covered trophozoite, extending a pseudopodium, moving across or through the mucus blanket.

Amoeba motility. As the HM-1 trophozoites appeared unable to penetrate the mucus blanket, we decided to study the effects of mucin, mucus, and the rat colon luminal environment on amoeba motility. Trophozoites were suspended in TYI or TYI-S medium. All three methods of scoring motility indicated that the addition of serum to TYI medium increased motility (Fig. 4a, right and left panels). This difference between motility in TYI and TYI-S allowed
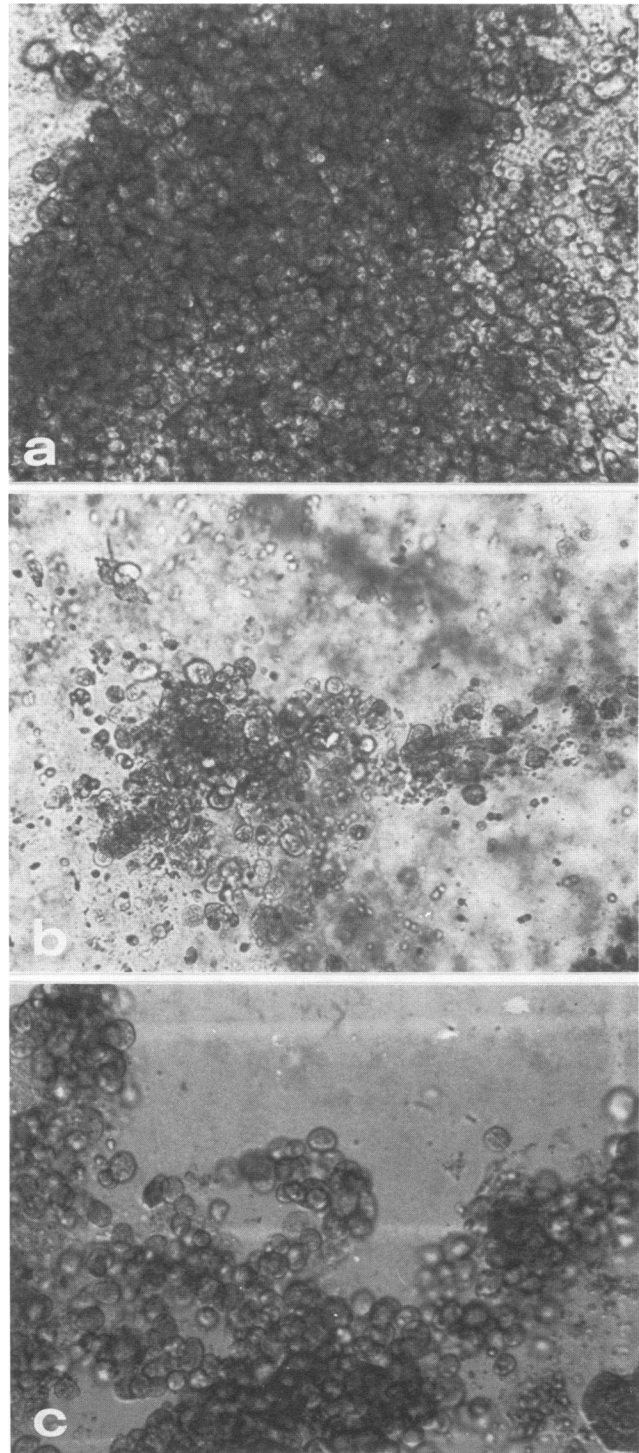

FIG. 2. *E. histolytica* trophozoites in TYI medium were inoculated into in vivo rat colon loops. The luminal fluid was removed at 5 min or 4 h, and the remaining mucus blanket was removed with cetylpyridinium bromide. This residual mucus blanket was rich in adsorbed trophozoites at 5 min (a), but fewer trophozoites were visible by 4 h (b). By 4 h, the trophozoites were instead found adsorbed to sloughed mucus blanket fragments that were removed with the luminal fluid (c).
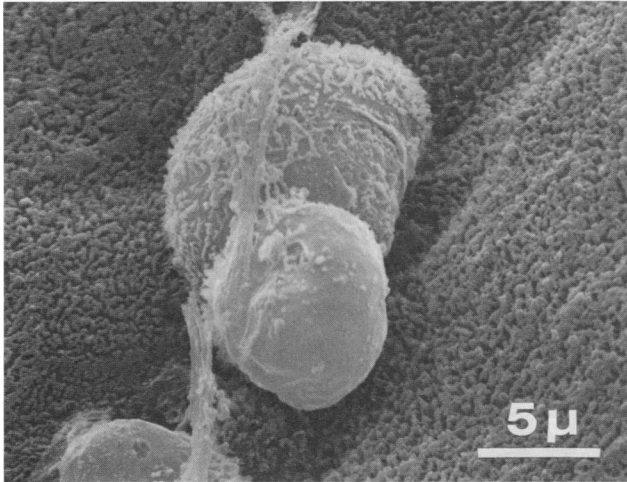
FIG. 3. E. histolytica trophozoites in the mucus blanket of in vivo rat colon. A pseudopodium is being formed by the amoeba, which is otherwise completely encased in mucus. This was a freeze-substituted preparation.

us a means of testing compounds for their ability to stimulate or inhibit trophozoite motility. Compounds could be tested for their ability to stimulate motility by suspending the amoebae in TYI, and suspending the amoebae in TYI-S allowed us to test a compound for its ability to inhibit motility. Points b and c in both sides of Fig. 4 also show the motility of Entamoeba trophozoites in TYI and TYI-S after their sojourn in the in vivo rat colon for 5 min. After recovery from the lumen, these trophozoites could be divided into two subgroups, those that were free in aqueous solution (points b in Fig. 4) and those that were associated with mucus, often in clumps (points c). Figure 5 shows the scanning electron microscopic appearance of such free (a) and mucus-associated (b) amoebae. The rat colon lumen apparently contained some component(s) that stimulated trophozoite motility, because when the amoebae were removed from the in vivo colon after a 5-min sojourn in TYI medium, the motility of the trophozoites that were free of mucus fragments was increased. The subpopulation that was associated with mucus fragments exhibited a reduced motility when the amoebae were inoculated in TYI-S medium.

Figure 6 shows the effect of a salivary mucin preparation (Sigma type I) on motility. For brevity, in this and subsequent figures, only the lines intersected data are shown. It is apparent from Fig. 6 that mucin inhibited motility in a dose-dependent way. None of the mucin concentrations used caused gel formation, as determined by direct observation or by measurement of apparent viscosity. The apparent viscosity of mucin at 6 mg/ml in TYI-S was 4.4 centipoise at a shear rate of 1.15 s^{-1}, measured with a cone-plate viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, Mass.) at 37°C. Intestinal mucus gels have an apparent viscosity in excess of 30 centipoise (14).

At low concentrations, this purified mucin preparation stimulated the motility of trophozoites suspended in TYI. This led us to test the effects of three major end-group carbohydrates of mucin oligosaccharides, N-acetyl-neuraminic acid, N-acetyl-D-galactosamine, and L-fucose, for their effects of trophozoite motility. N-Acetyl-D-galactosamine (1 mM) had no effect on motility. Figure 7 summarizes the sialic acid and L-fucose data. The sialic acid preparation tested, N-acetyl-neuraminic acid (Sigma type IV), stimu-

lated motility, even at a concentration of 0.2 mM in TYI. On the other hand, L-fucose (0.2 mM) in TYI-S inhibited motility. There was a weak stimulatory effect of a higher L-fucose concentration (1 mM) in TYI.

As Entamoeba trophozoite motility was increased by the presence of serum in TYI (i.e., TYI-S) and the presence of rat colon solutes in TYI, we studied the effects of both of these conditions on erythrocyte phagocytosis. Neither the presence of serum in TYI nor exposure of TYI to rat colon lumen for 5 min significantly increased subsequent phagocytosis.

DISCUSSION

When E. histolytica trophozoites were inoculated into in vivo rat colon loops, they appeared to be rapidly absorbed to the luminal mucus (Fig. 1 and 2a). The motility of those trophozoites that escaped such adsorption was high, apparently being stimulated by some component(s) elaborated by, or found in, the colon (Fig. 4b), whereas the motility of the trophozoites adsorbed to mucus was almost undetectable (Fig. 4c). However, amoebae adsorbed to, or encased in, mucus were still able to extend pseudopodia (Fig. 3 and 5b).

The mucus-induced reduction in trophozoite motility may have been due both to a chemical effect or signal and to physical retardation caused by the mucus gel itself. A
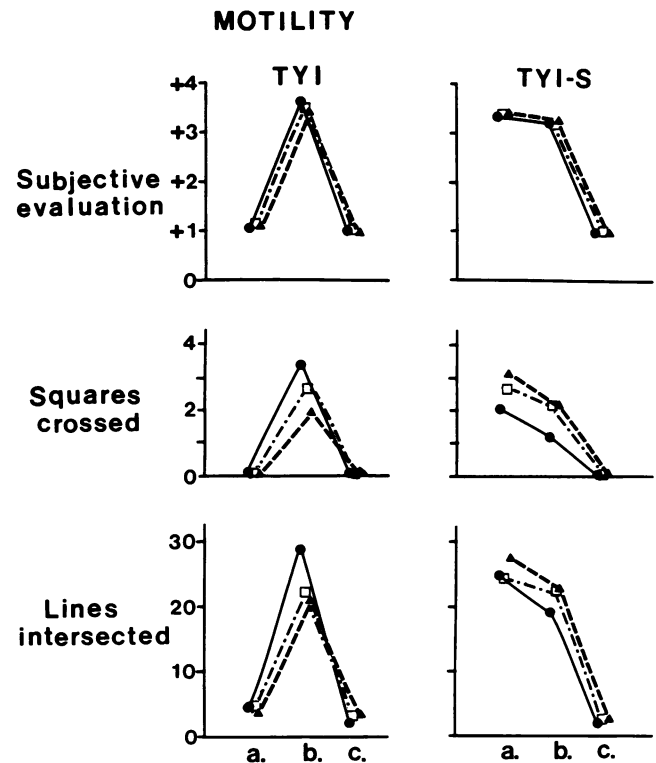

FIG. 4. Three methods were used to evaluate the motility of E. histolytica trophozoites maintained in either TYI medium or TYI-S medium. Amoebae were suspended in the appropriate media and their motility was measured (a), or they were inoculated into an in vivo rat colon loop and removed 5 min later (b and c). These amoebae were either free from (b), or obviously associated with (c), fragments of intestinal mucus. Three complete sets of experiments are shown with each medium. Each point represents the mean motility value of 10 trophozoites.
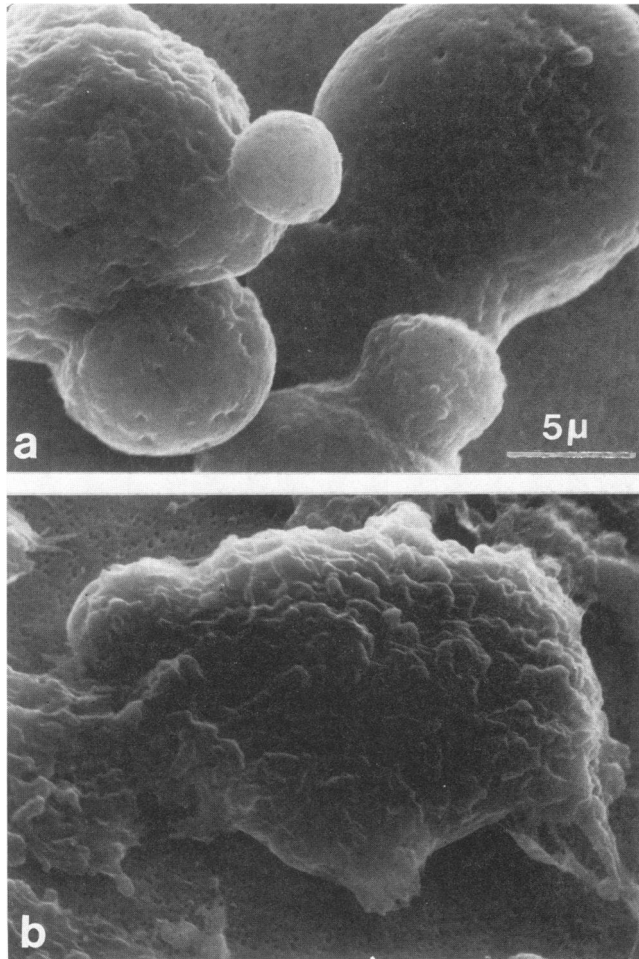
FIG. 5. *E. histolytica* trophozoites inoculated into in vivo rat colon and removed 5 min later. Amoebae were recovered either free of (a), or associated with (b), mucus. This was a glutaraldehyde-fixed preparation.

chemical factor is suggested by the observation that salivary mucin inhibited motility, even though the mucin was in a sol state (Fig. 6). The mucus blanket is a rich source of potential chemical signals. In addition to entrapped bacteria and sloughed epithelial cells, two of three mucin carbohydrates tested affected amoeba motility. *N*-Acetyl-neuraminic acid stimulated motility at concentrations as low as 0.2 mM, and
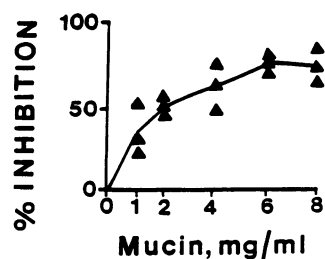

FIG. 6. Percent inhibition of *E. histolytica* trophozoite motility (measured as the number of lines intersected) plotted as a function of the concentration of a salivary mucin preparation in TYI-S medium.
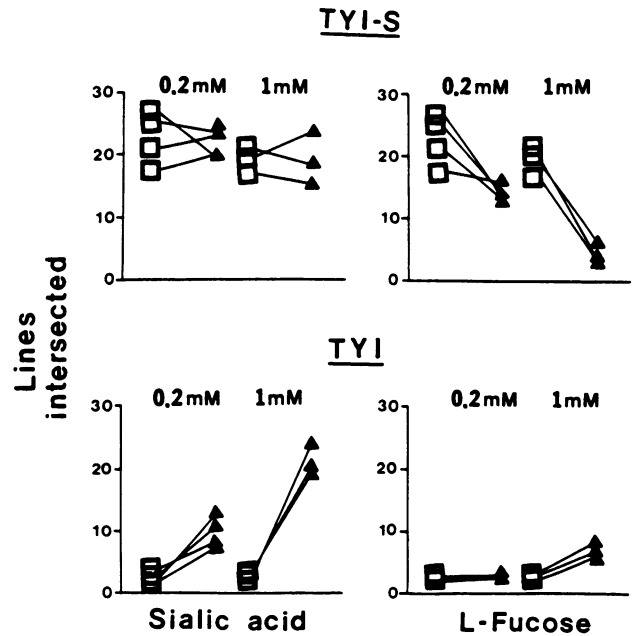

FIG. 7. The motility of *E. histolytica* trophozoites was measured in TYI-S or TYI in the presence or absence of either *N*-acetylneuraminic acid or L-fucose, using the lines intersected method. The basal trophozoite motility was high in TYI-S, so this medium was used in assessing motility inhibition, whereas the basal motility was low in TYI, and this medium was used in assessing motility stimulation. Open squares represent medium without the test carbohydrate; closed triangles represent medium containing the test carbohydrate.

L-fucose inhibited motility at the same concentration. *N*-Acetyl-D-galactosamine had no effect at a concentration of 1 mM. These carbohydrates constitute major terminal sugars of mucin oligosaccharides (20) and would therefore be potentially available to modify *Entamoeba* trophozoite motility in the intestine.

In our hands, experimental amebiasis could not be produced in adult male Sprague-Dawley rats by the intracecal inoculation of axenically cultivated HM-1 trophozoites. Mucus has been postulated to provide the gastrointestinal mucosa with a protective microenvironment (4), and the study reported here suggests that the mucus blanket effectively acted as a barrier to the access of amoebae to the mucosal epithelium. Mirelman and co-workers (12) also found that clear rabbit colon mucus inhibited attachment of *Entamoeba* trophozoites to mammalian cells.

The present study raises the possibility that both chemical signals and physical forces control amoeba motility in the intestinal lumen. Smith and Meerovitch (21) have shown that *E. histolytica* trophozoites can penetrate a mechanical barrier by generating a force of 3.3×10^{-6} N. This may be sufficient to break through a mucus blanket. On the other hand, if there is no chemotropic stimulus encouraging the trophozoites through the entire depth of the blanket, then the expenditure of such energy may not occur. In fact, the luminal surface of the mucus blanket may be a very attractive location at which to remain, due to the presence of bacteria and sloughed epithelial cells.

LITERATURE CITED

1. **Bos, H. J.** 1979. *Entamoeba histolytica*: cytopathogenicity of intact amoebae and cell-free extracts; isolation and characterization of an intracellular toxin. Exp. Parasitol. **47**:369–377.

2. **Brandt, H., and R. P. Tamayo.** 1970. Pathology of human amebiasis. Human Pathol. **1**:351–385.

3. **Diamond, L. S., D. Harlow, and C. C. Cummick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoeba. Trans. R. Soc. Trop. Med. Hyg. **4**:431–432.

4. **Florey, H.** 1962. The secretion and function of intestinal mucus. Gastroenterology **42**:326–329.

5. **Knight, R., R. G. Bud, and T. F. McCaul.** 1975. Fine structural changes at *Entamoeba histolytica* rabbit kidney cell (RK 13) interface. Ann. Trop. Med. Parasitol. **69**:197–202.

6. **Kobiler, D., and D. Mirelman.** 1980. Lectin activity in *Entamoeba histolytica* trophozoites. Infect. Immun. **29**:221–225.

7. **Lushbaugh, W. B., A. B. Kairalla, J. R. Cantey, A. F. Hofbauer, and F. E. Pittman.** 1979. Isolation of cytotoxin-enterotoxin from *Entamoeba histolytica*. J. Infect. Dis. **139**:9–17.

8. **Lushbaugh, W. B., A. B. Kairalla, A. F. Hofbauer, J. R. Cantey, and F. E. Pittman.** 1980. Further studies on a cytotoxin/enterotoxin from *Entamoeba histolytica*. Arch. Invest. Med. **11**:129–133.

9. **Mattern, C. F. T., D. B. Keister, and R. C. Natovitz.** 1980. *Entamoeba histolytica* "toxin", fetuin neutralizable and lectin-like. Am. J. Trop. Med. Hyg. **29**:26–30.

10. **McGowan, K., C. F. Deneke, G. M. Thorne, and S. L. Gorbach.** 1982. *Entamoeba histolytica* cytotoxin: purification, characterization, strain virulence, and protease activity. J. Infect. Dis. **146**:616–625.

11. **McGowan, K., A. Kane, N. Asarkof, J. Wicks, V. Guerina, J. Kellum, S. Baron, A. R. Gintzler, and M. Donowitz.** 1983. *Entamoeba histolytica* causes intestinal secretion: role of serotonin. Science **221**:762–763.

12. **Mirelman, D., C. Feingold, A. Wexler, and R. Bracha.** 1983. Interaction between *Entamoeba histolytica*, bacteria and intestinal cells. Ciba Found. Symp. **99**:2–30.

13. **Neal, R. A., and W. G. Harris.** 1975. Attempts to infect inbred strains of rats and mice with *Entamoeba histolytica*. Trans. R. Soc. Trop. Med. Hyg. **69**:429–430.

14. **Njoku, O. O., and G. J. Leitch.** 1983. Separation of cholera enterotoxin-induced mucus secretion from electrolyte secretion in rabbit ileum by acetazolamide, colchicine, cycloheximide, cytochalasin B and indomethacin. Digestion **27**:174–184.

15. **Orozco, E., S. F. Castelo, and A. Martinez-Palomo.** 1982. Deteccion del dano celular temprano producido por *Entamoeba histolytica* sobre epitelios. Arch. Invest. Med. **13**:169–175.

16. **Pamer, T., G. B. Jerzy Glass, and M. I. Horowitz.** 1968. Purification and characterization of sulfated glycoproteins and hyaluronidase-resistant mucopolysaccharides from dog gastric mucosa. Biochemistry **7**:3821–3829.

17. **Peters, R. S., N. Gitlin, and R. D. Libke.** 1981. Amebic liver abscess. Annu. Rev. Med. **32**:161–174.

18. **Ravdin, J. I., B. Y. Croft, and R. L. Guerrant.** 1980. Cytopathogenic mechanisms of *Entamoeba histolytica*. J. Exp. Med. **152**:377–390.

19. **Ravdin, J. I., and R. L. Guerrant.** 1982. Separation of adherence, cytolytic and phagocytic events in the cytopathogenic mechanisms of *Entamoeba histolytica*. Arch. Invest. Med. **13**:123–128.

20. **Silberberg, A., and F. A. Meyer.** 1982. Structure and function of mucus. Adv. Exp. Med. Biol. **144**:53–74.

21. **Smith, J. M., and E. Meerovitch.** 1982. *Entamoeba histolytica*, the dynamics of penetrating a meniscus. Can. J. Zool. **60**: 2124–2128.

22. **Takeuchi, A., and B. P. Phillips.** 1975. Electron microscopic studies of experimental *Entamoeba histolytica* infection in the guinea pig. I. Penetration of the intestinal epithelium by trophozoites. Am. J. Trop. Med. Hyg. **24**:34–48.

23. **Trissl, D., A. Martinez-Palomo, M. de la Torre, R. de la Hoz, and P. Suarez.** 1978. Surface properties of *Entamoeba*: increased rates of human erythrocyte phagocytosis in pathogenic strains. J. Exp. Med. **148**:1137–1145.

24. **Trissl, D. A., A. Martinez-Palomo, C. Arguello, M. de la Torre, and R. de la Hoz.** 1977. Surface properties related to concanavalin A-induced agglutination. A comparative study of several amoeba strains. J. Exp. Med. **145**:652–665.

25. **Udezulu, I. A., G. J. Leitch, and G. B. Bailey.** 1982. Use of indomethacin to demonstrate enterotoxic activity in extracts of *Entamoeba histolytica* trophozoites. Infect. Immun. **36**:795–801.