Cytopathology of parasitic disease

Ciba Foundation symposium 99

Organized by the Ciba Foundation, London, UK, in partnership with the Centro Médico Docente La Trinidad, Caracas, Venezuela

1983

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Chairman's introduction

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The cytopathology of parasitic disease is an exceedingly important subject today because the field has really gained momentum in the past 10 years, and our knowledge about host–parasite interaction at the chemical and immunological level is increasing rapidly. Most of the people here have played a major part in the development of this work. I have previously had the pleasure of attending another Ciba Foundation meeting, in London—Parasites in the immunized host: mechanisms of survival (Ciba Found Symp 25). The discussion at that meeting almost predicted one of the major areas of parasite cytopathology today—i.e. how the parasite evades the host's responses. I hope that this meeting will be as influential on future research as that one has been. This is the second Ciba Foundation meeting held in Venezuela, the first one being in 1973, on Trypanosomiasis and leishmaniasis, with special reference to Chagas' disease (Ciba Found Symp 20). Participants here come from Europe, USA, Venezuela, Brazil, Kenya, Mexico and Israel. Thus, both the site of the meeting and the countries of those attending it attest to the truly international nature of the Ciba Foundation. It is a great honour for me to be invited to chair this meeting, and I welcome the opportunity that it provides for cooperation between the Centro Médico Docente La Trinidad and the Ciba Foundation.

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Interactions between *Entamoeba histolytica*, bacteria and intestinal cells

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Abstract Axenically grown pathogenic and non-pathogenic isolates of *Entamoeba histolytica* have been shown to adhere to mammalian epithelial cells and bacteria by virtue of carbohydrate-binding proteins present on their cell surfaces. The interaction of amoeba isolates of low pathogenicity with a variety of gram-negative bacteria, mainly *Escherichia coli* strains which are readily ingested by the amoebae after relatively short periods, significantly increased the ability of the trophozoites to: (a) destroy and ingest intestinal epithelial cells; (b) secrete a cytopathic substance which morphologically affects a variety of tissue-cultured cells; and (c) cause hepatic abscesses in hamsters. Addition of carbohydrates that inhibit the lectin-mediated attachment of bacteria to amoebae prevented the enhancement of virulence. Interaction of the amoebae with bacteria that were heat-inactivated, glutaraldehyde-fixed or disrupted by sonication, as well as with bacteria precoated with antibodies or concanavalin A, did not lead to an increase in virulence. Moreover, short prior treatments of the bacteria with inhibitors of protein synthesis, but not with cell-wall synthesis inhibitors, also prevented the stimulation. The results indicate that interactions of amoebae with certain bacteria may be responsible for the increase in amoebic virulence.

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The study of microbial interrelationships as an aspect of microbial pathogenicity is an obscure area of microbiology which has lent itself to only limited scientific investigation. It is generally recognized that the virulence of *Entamoeba histolytica* is related in part to association of the amoebae with suitable bacterial species. Early observations by Westphal (1937), Phillips & Gorstein (1966), Wittner & Rosenbaum (1970) and others have demonstrated that association of bacteria with amoebae augments the virulence of the amoebae as determined by their ability to cause lesions when inoculated into animals. Furthermore, since amoebic symptoms sometimes seem to disappear from the intestine after treatment with bactericidal but non-amoebicidal

drugs such as β-lactams and sulpha-derivatives, this suggests that amoebic pathogenicity may depend on some factor(s) inherent in the interaction of amoebae with certain microorganisms.

Attachment to and ingestion of bacteria by trophozoites of *E. histolytica*

The development of *in vitro* axenic cultivation methods for *E. histolytica* by Diamond (1968) and Diamond et al (1978) has made it possible to obtain amoebic inocula free from all other microorganisms with which *E. histolytica* is usually associated in the intestine, and has facilitated the study of its interactions with bacteria.

Since surfaces of trophozoites of *E. histolytica* have been shown to contain concanavalin A receptors (Trissl et al 1977), it was logical to assume that the adherence to them by bacteria that possess mannose-binding lectins (Ofek et al 1977) would be similar to that observed for macrophages (Bar-Shavit et al 1977, 1980). Amoebae were found to be very selective in their interaction with bacteria. The attachment to the amoebae of organisms that possess organelles with mannose-binding properties, such as *Escherichia coli* 7343 or *E. coli* serotype 075, depended on time and concentration of the organisms. The average number of bacteria that attached per trophozoite at high multiplicity (1:1000) was between 40 and 100. The adherence of *E. coli* 7343 was found to be markedly inhibited by the presence of α-methylmannoside (α-MM) (Fig. 1). Precoating of the mannose receptors of the amoebae by concanavalin A also markedly blocked the attachment of *E. coli* cells (Fig. 1). Attachment of bacteria to the amoebae was mostly irreversible, and later addition of α-MM did not remove the bacteria. Only when low temperatures (5 °C) or glutaraldehyde-fixed amoebae were used—conditions that prevent ingestion—could removal of the attached bacteria by α-MM be achieved. These results implied that, after initial interaction, the amoebae began internalizing the bacteria. Further evidence for this was obtained from electron microscopic examination of thin sections (Fig. 2) as well as from the observation that phagocytized bacteria became very sensitive to solubilization by Triton X-100 (0.2%), whereas bacteria that remained attached to the surface (in incubations done in the cold) were not sensitive to the detergent (Bracha et al 1982).

Trophozoites of *E. histolytica* were found to shun bacteria that did not have a capacity to bind to mannose-containing surfaces. The only types of non-mannose binding bacteria that were found attached to trophozoites were *E. coli*, serotype 055, and *Salmonella*, serotype 050. The attachment of these bacteria was sensitive to galactose and *N*-acetylgalactosamine, as well as to a lipopolysaccharide preparation from *E. coli* 055, but was insensitive to α-MM

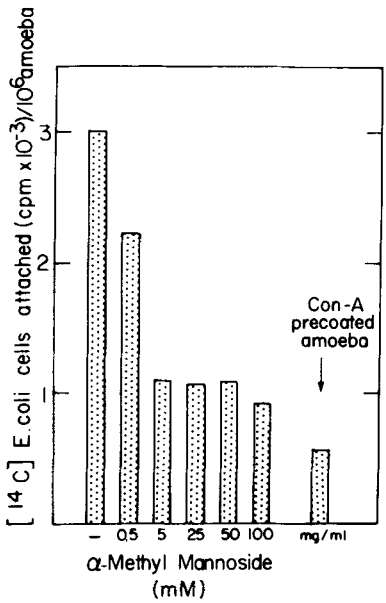

FIG. 1. Effect of increasing concentrations of α-methylmannoside and precoating of trophozoites with concanavalin A (Con-A) on the adherence of *E. coli* 7343 to amoebae. The reaction was carried out for 15 min at 37 °C. cpm, counts per minute

(Table 1). The attachment and ingestion of *E. coli* 055 was not inhibited by mannose-binding bacteria (*E. coli* 7343), but no adherence occurred with glutaraldehyde-fixed amoebae, or in the cold (5 °C). However, in contrast to *E. coli* 7343, no major differences were observed when heat-inactivated *E. coli* 055 (100 °C, 15 min) were used. Furthermore, periodate treatment of bacteria markedly affected their ability to bind to amoebae. These findings indicate that for *E. coli* 055 the interaction is mediated by an amoebic lectin (Kobiler & Mirelman 1980, Ravdin & Guerrant 1981) which recognizes a distinct carbohydrate on the bacterial lipopolysaccharide.

Other types of bacteria such as *Shigellae* species or gram-positives like *Staphylococcus aureus* and *Micrococcus luteus*, which do not have surface lectins, did not attach or interact with amoebae. On the other hand, bacteria opsonized with specific rabbit immune serum were readily attached to and ingested by the trophozoite. In contrast to peritoneal macrophages (Koide et al 1977), the attachment to the amoebae was not mediated by Fc receptors because *Shigella* precoated only with Fab' dimers of anti-*Shigella* immunoglobulin were similarly attached (Bracha et al 1982). Adherence of opsonized *Shigella* was blocked by carbohydrates similarly to the inhibition of *E. coli* 055

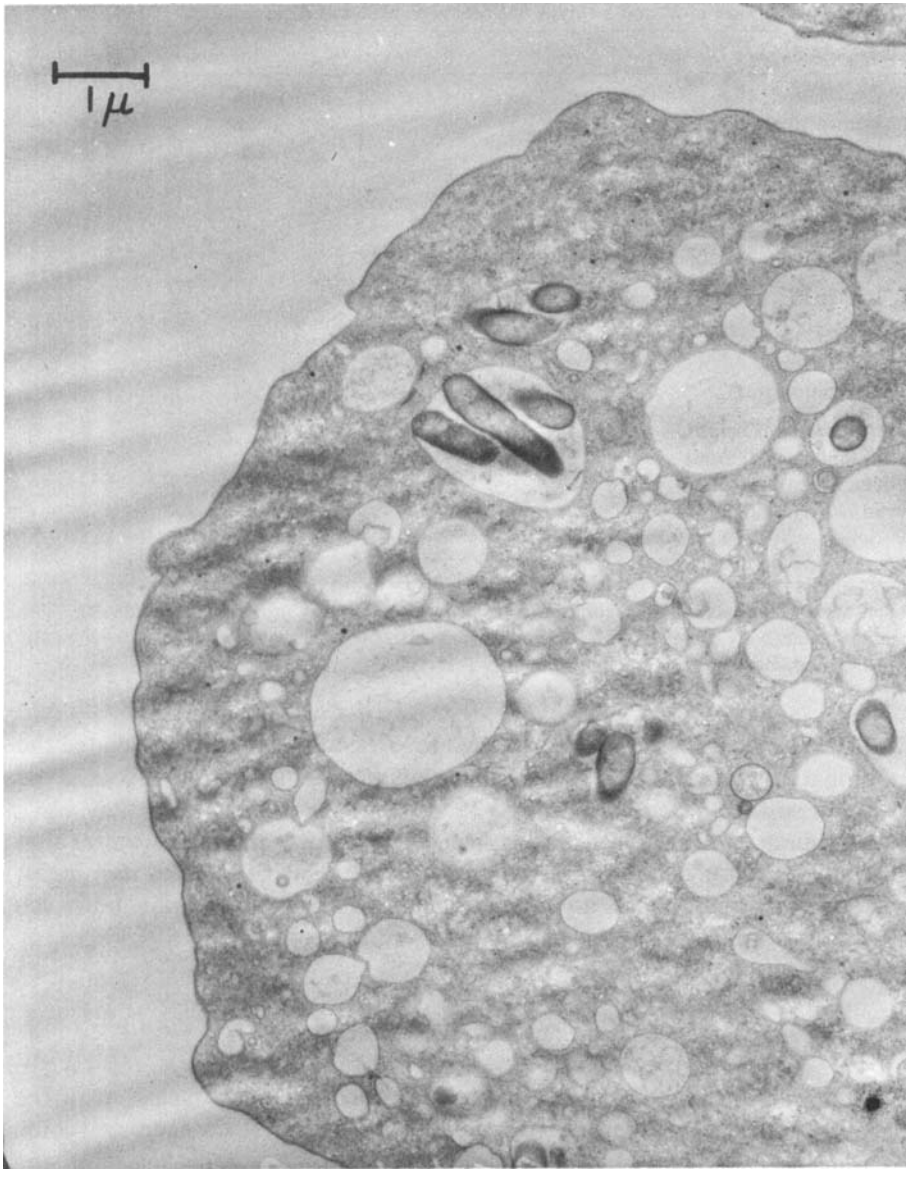

FIG. 2. Electron micrograph of thin section of an amoeba that interacted for 30 min at 37 °C with *E. coli* 7343. Bacteria were found almost exclusively inside vacuoles.

TABLE 1 Adherence of *E. coli* serotype 055[b] to trophozoites of *E. histolytica*

Variable Additions	*Attachment[a] %*
None	100
Galactose 10 mg/ml	41
α-methylmannoside 10 mg/ml	106
$GlcNAc_{2-3}$ 2 mg/ml	80
Lactose 10 mg/ml	43
GalNAc 10 mg/ml	42
Lipopolysaccharide (*E. coli* 055) (4 mg/ml)	48
Lipopolysaccharide (*E. coli* 7343) (4 mg/ml)	115
E. coli 7343 (2.5×10^8)	83
E. coli 7343 + α-MM	89
Heparin mg/ml	94
Asialofetuin mg/ml	54
Pretreatments of bacteria	
γ-irradiation (500 krad)	110
Glutaraldehyde fixation	94
Heat inactivation	89
Periodate oxidation (25 mM, 10 min)	52
Glutaraldehyde-fixed amoebae	<5

[a]The number of bacteria that adhered to each trophozoite was usually between 25–50. [b]Very similar results were obtained with *Salmonella greenside* 050 instead of *E. coli* 055. A ratio of 1000 ^{14}C-labelled bacteria per amoeba was used.

(Table 1), indicating that in these cases, too, the amoebic lectin mediated the binding to carbohydrates likely to be on the immunoglobulins that coated the bacterial surface (Niedermeier et al 1971) (Fig. 3).

In summary, our studies have shown that *E. histolytica* trophozoites that inhabit the colon have a variety of options by which to attach and ingest bacteria. Our main efforts are now directed towards understanding how such interactions might affect the pathogenicity of the amoebae.

Interactions between trophozoites and epithelial cells

The initial step in the amoebic infectious process is the attachment of the parasite to, and its proliferation on, mucosal surfaces of the caecum and colon (Chevez et al 1976). In many cases this is followed by invasion and penetration of the amoebae through the intestinal wall, leading to secondary but more serious infections such as abscesses of the liver, brain and lung (Martinez-Palomo 1982).

Carbohydrate-binding proteins on its cell surface mediate the adherence of *E. histolytica* to mammalian cell surfaces. Depending on the source of target

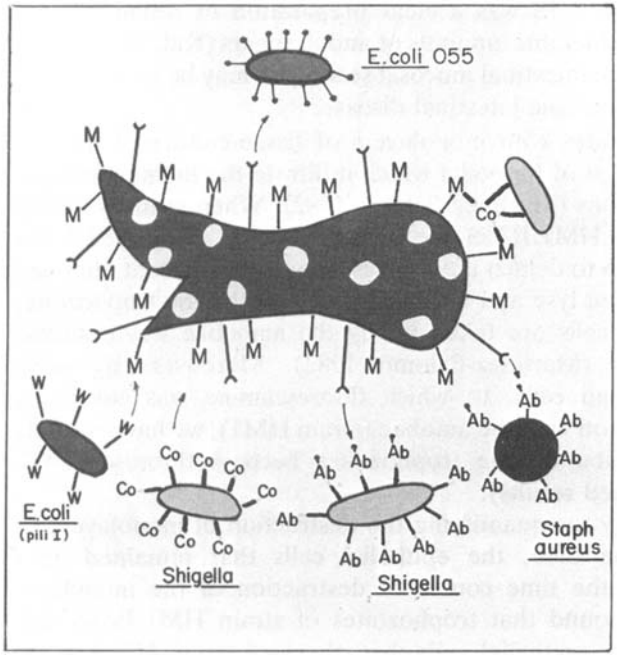

FIG. 3. Summary of various possibilities for attachment of different bacteria to the amoeba trophozoites. M, mannose-containing receptors on the amoeba surface; ———C, a carbohydrate-binding (lectin) activity; W, surface pili, on *E. coli*, which have mannose-binding properties; Co, concanavalin A bound to receptors on the bacteria; Ab, antibodies; and ———●, *N*-acetylgalactosamine or galactose moieties on bacterial surfaces or on opsonins.

cells and conditions of the assay, two mechanisms of adherence have been detected. Adherence of amoebae to a human intestinal cell line (Henle 407), to baby hamster kidney (BHK) cells (Kobiler & Mirelman 1980) and to erythrocytes (Orozco et al 1982) was specifically inhibited by oligosaccharides of *N*-acetylglucosamine $(GlcNAc)_{3-4}$, or by purified bacterial peptidoglycan preparations, as well as by precoating of the mammalian cells with wheat-germ agglutinin. Adherence of amoebae to Chinese hamster ovary (CHO) cells (Ravdin & Guerrant 1981), as well as to *E. coli* serotype 055 (see above), was inhibited by *N*-acetylgalactosamine (GalNAc) and galactose.

No significant differences in the adherence capability of various isolates of *E. histolytica* were detected (Kobiler & Mirelman 1980). The inhibitory potency of GlcNAc- or GalNAc-containing molecules implies that the mammalian cell receptor may be a surface component, probably a glycoprotein, with structures that contain such carbohydrate moieties (Yamashita et al 1978, Mizuochi et al 1978). Another potent inhibitor of trophozoite

attachment to mammalian cells was a clear preparation of rabbit colonic mucus that contained considerable amounts of amino sugars (Katz et al 1982). The inhibitory action of the intestinal mucosal secretions may be important in the development of the amoebic intestinal disease.

Incubation of trophozoites with monolayers of tissue-cultured epithelial cells leads to the formation of filopodia which infiltrate the mammalian cell and form stable connections (Martinez-Palomo 1982). When virulent strains of *E. histolytica* such as HM1:IMSS are incubated with an epithelial cell monolayer, the cells begin to detach themselves from their adjacent ones and to round up; some of them lyse and others are ingested by the trophozoite. Evidence that epithelial cells are taken up by the amoebae was obtained by electron microscopy (Martinez-Palomo 1982). Moreover, by using monolayers of mammalian cells, to which fluorescamine was covalently bound before the incubation with the amobae (strain HM1), we have recently observed that after incubation the trophozoites become fluorescent (C. Feingold et al, unpublished results).

We developed an assay for quantifying the destruction of monolayers by staining, with methylene blue, the epithelial cells that remained after incubation. We studied the time course of destruction of the monolayer (5×10^5 cells/well) and found that trophozoites of strain HM1:IMSS destroyed considerably more epithelial cells than those of strain HK-9 in the same conditions (Table 2). An increased number of trophozoites per well markedly increased the rate of monolayer destruction. Inhibition of monolayer destruction was observed with a number of carbohydrates such as galactose and lactose (>85% at 10 mg/ml) as well as with serum (1%) and rabbit colonic mucus (7 mg/ml) (Katz et al 1982).

TABLE 2 Destruction of tissue-cultured monolayers by *Entamoeba histolytica* trophozoites

E. histolytica strain	*Interaction with bacteria*	*Destruction of monolayer (%)*	
		(Expt 1)	*(Expt 2)*
HM1 : IMSS	None	31 ± 1	80 ± 3
HM1 : IMSS	*E. coli* 7343	64 ± 3	–
HM1 : IMSS	*E. coli* 055	–	84 ± 6
HK-9	None	12 ± 1	19 ± 3
HK-9	*E. coli* 7343	65 ± 3	–
HK-9	*E. coli* 055	–	49 ± 1

Interaction was achieved by suspending trophozoites (4×10^5 in 0.2 ml Eagle's minimal media) in wells (0.5 cm diameter) containing a confluent monolayer of baby hamster kidney cells (5×10^5) for 60 min at 37 °C. An overnight growth of *E. coli* cells was incubated with trophozoites at a multiplicity of 1000 : 1 for 15 min at 37 °C, before their addition to the wells, and then further incubated for 60 min. Destruction of monolayers was measured by methylene blue staining of the mammalian cells that remained in the wells after the interaction. –, not measured.

Augmentation of *E. histolytica* virulence on association with bacteria

The reassociation of axenically grown amoebae with an undefined *E. coli* strain was found to restore virulence, as shown by an increase in hepatic abscess formation in the hamster model (Wittner & Rosenbaum 1970). Moreover, monoxenic strains maintained together with the protozoan flagellate *Crithidia fasciculata* or with a mixed species of *Proteus*, *Klebsiella* and *E. coli* had a significantly higher ability to form hepatic abscesses in hamsters (Bos & Hage 1975). The duration of restored virulence was relatively short, and removal of the bacteria reduced the amoebic virulence. On the other hand, passage of axenically grown amoebae through a liver and reisolation from the liver augmented their virulence, and this could be maintained in axenic media for several months (Bos & Hage 1975).

The assay for monolayer destruction was used to evaluate the effect of bacteria on the virulence of amoeba strains. Preincubation of trophozoites of strain HK-9 (2×10^5 cells/well) with varying amounts of *E. coli* 7343 (a bacterium with mannose-binding properties) gradually increased the rate of monolayer destruction. Maximal destruction rates were observed when the ratio between trophozoites and bacteria was 1 : 1000 (Fig. 4), although a 20% increase in the rate of destruction could be seen even at 1 : 10 ratios. Addition

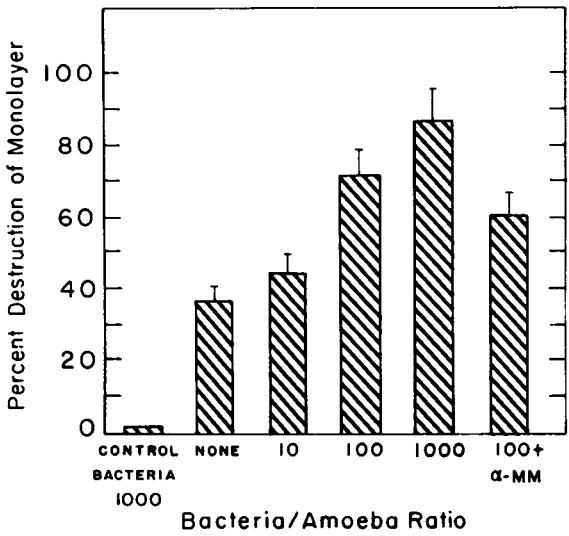

FIG. 4. Interaction of amoebae with bacteria, and augmentation of amoebic virulence. Destruction of tissue-culture monolayer by trophozoites of *E. histolytica* (strain HK-9) after interaction with cells of *E. coli* 7343 at different multiplicities. α-MM = α-methylmannoside (1%).

of α-MM (1%) to a bacteria–amoebae mixture (at 1 : 100 ratio) to provide conditions that inhibit by approximately 90% the attachment of bacteria to the amoebae, inhibited the increase in virulence (Fig. 4). Longer periods of trophozoite preincubation with bacteria did not markedly increase further the amoebic virulence. When strains HM1 : IMSS and HK-9 were compared after interaction with bacteria, the virulence increased in both, but was proportionately higher for strain HK-9 (Table 2).

Increased amoebic virulence was observed after interaction not only with *E. coli* 7343 but also with other *E. coli* strains that possessed mannose-binding activity (Table 3), as well as with strain *E. coli* 055. Mannose-binding *E. coli* strains that were cured for plasmids or were known not to contain plasmids (strains CR63 or CA274, Table 3) augmented the amoebic virulence, but *Shigella* strains and bacterial cells such as *E. coli* 09 : K(29)$^+$: H$^+$ that did not adhere to the amoebae did not increase the virulence. Moreover, the opsonization of *Shigella flexneri*, which we had previously shown to facilitate their ingestion by the trophozoite, did not augment the virulence of HK-9 strains (Table 3).

TABLE 3 Bacterial strains that augment amoebic virulence

Strain	*Adherence to amoebae*	*Specificity of adherence*	*Augmentation of virulence*
E. coli 055	3+	Gal,GalNAc	3+
E. coli 075	3+	Man	3+
E. coli 7343	3+	Man	3+
E. coli CR63 (no plasmids)	2+	Man	2+
E. coli CA274 (no plasmids)	2+	Man	3+
E. coli 09 : K$^-$29 : H$^-$	0	–	0
E. coli 09 : K$^+$29 : H$^+$	0	–	0
Serratia marcescens	3+	Man	3+
Shigella flexneri	0	–	0
Shigella flexneri (ops)	3+	Gal,GalNAc	0
Staphylococcus aureus	0	–	0
Staphylococcus aureus (ops)	3+	Gal,GalNAc	0

For experimental conditions, and the results of a marked (3+) augmentation with *E. histolytica* strain HK-9, see Table 2. —, no adherence observed.
Gal, galactose; GalNAc, *N*-acetylgalactosamine; Man, mannose.

For virulence to be stimulated, the bacteria had to be intact. Sonicates of bacteria or heat-inactivated *E. coli* 7343 did not augment the virulence (Table 4). On the other hand, bacteria exposed to 500 krad from a cobalt X-ray source (after which no capability to form colonies remained) could still enhance the virulence. Bacteria whose growth was blocked by short (1 h)

TABLE 4 Effect of pretreatments of bacteria on their ability to increase amoebic virulence

Pretreatment	*Augmentation*
None (log or stationary phase)	3+
Glutaraldehyde fixation	0
Heat-inactivation (15 min, 100 °C)	0
Sonication	0
γ-irradiation (99.9%, LD 2×10^4 rad)	3+
γ-irradiation (100%, LD 10^5 rad)	2+
γ-irradiation (100%, LD 5×10^5 rad)	1+
Growth with inhibitors of cell-wall synthesis	
(bicozamycin 200 μg/ml, 2 h)	3+
(cephalexin 8 μg/ml, 1 h)	3+
Growth with inhibitor of protein synthesis	
(amikacin 100 μg/ml, 2 h)	0

For experimental conditions, and results of marked (3+) augmentation with *E. histolytica* strain HK-9, see Table 2. The experiments were done with both *E. coli* 7343 and *E. coli* 055.
LD, lethal dose.

pretreatments with β-lactams or bicozamycin were capable of stimulating virulence, but amikacin-treated bacteria were not. Furthermore, the presence of galactose (1%) markedly inhibited all monolayer destruction.

In spite of their striking lytic ability, trophozoites of *E. histolytica* do not seem to penetrate the intact intestinal mucosa easily. In experimental *in vivo* and *in vitro* models, in which the interaction between trophozoites and the caecal mucosa of the guinea-pig was analysed, the initial penetration of amoebae was limited to the interglandular regions (Takeuchi & Phillips 1975, Mora Galindo et al 1978). These areas are usually where dying cells are continuously being sloughed off. In experiments done in our laboratory, interaction of trophozoites of strain HM1 : IMSS with guinea-pig colon sections (from the transverse region) did not damage the mucosal lining. On the other hand, trophozoites that had been reassociated with *E. coli* serotype 075 displayed a remarkable ability to penetrate and damage the mucosal lining (Fig. 5). Needless to say, incubations with the bacteria alone had no effect whatsoever. The sites of penetration again appeared to be in the interglandular zones. No damage to the mucosal lining was observed, however, when incubations were done in the presence of galactose (1%). Furthermore, there was very little damage to the mucosa when bacteria-stimulated trophozoites were incubated with sections of ascending colon.

Cytopathic effects of cell-free extracts of *E. histolytica*

Early attempts to demonstrate a trophozoite cell-free toxin were unsuccessful, apparently because of the use of serum in the culture medium (Bos 1979,

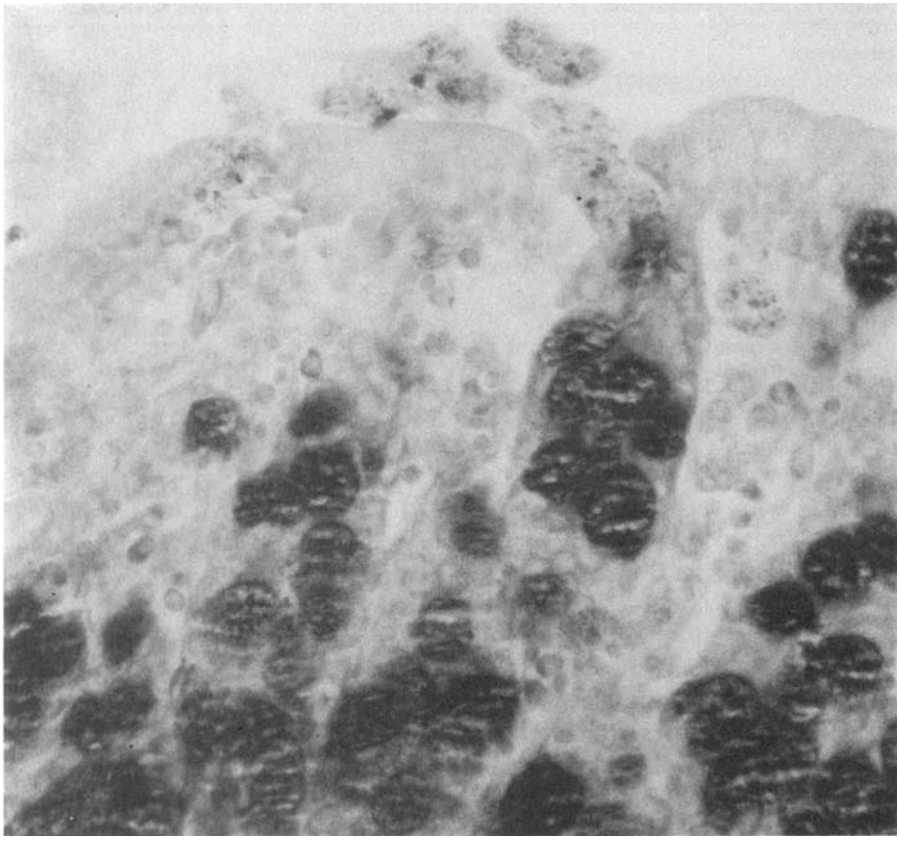

FIG. 5. Thin section of guinea-pig transverse colon, stained with Giemsa, after interaction with *E. histolytica* trophozoites that had been reassociated with *E. coli*, serotype 075. Trophozoites, in which bacteria can be seen, are penetrating between the mucosal cells into the tissue.

Lushbaugh et al 1979). Later studies have shown that the soluble fraction obtained from homogenates of pathogenic strains of amoebae possesses a substance that is toxic for a variety of mammalian cells (Lushbaugh et al 1979, 1981, Bos 1979, Ravdin & Guerrant 1981). The most commonly observed effect is the rounding up and detachment of tissue-cultured cells (Fig. 6). Some investigators have reported that amoeba homogenates can cause secretion of fluids similar to bacterial toxins in rabbit ileal loops (Lushbaugh et al 1979). This effect was demonstrated most notably in rabbits treated with indomethacin before the intraintestinal administration of the amoeba homogenate (Uedzulu et al 1982). The exact nature and mechanism of action

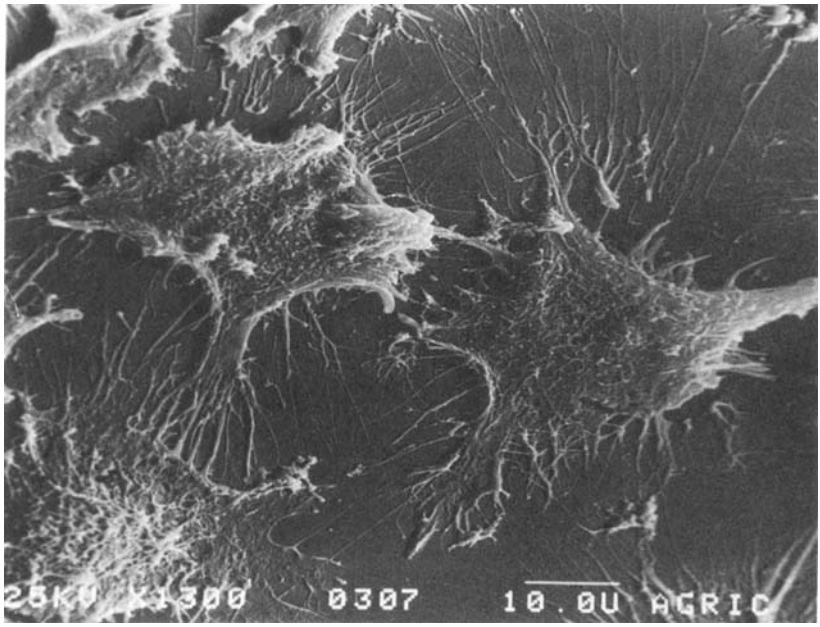

FIG. 6. Scanning electron micrograph of tissue-cultured human intestinal cells (Henle 407) after a short period of incubation with the soluble fraction of homogenates of *E. histolytica* trophozoites (strain HM1 : IMSS).

of the amoebic toxic substance is not yet completely clear, and may be the additive result of a number of biological activities. Amoebae, and particularly the pathogenic strains, have numerous proteolytic activities including collagenases (Muñoz et al 1982, Gadasi & Kessler 1983), phospholipases (J. I. Ravdin & R. L. Guerrant in Abstracts of the 22nd interscience conference on antimicrobial agents and chemotherapy, p 30, 1982, Miami) and a pore-forming ionophore type of material (Rosenberg et al 1982), all of which may affect host cells, and the intestinal mucosa in particular.

Using the above-mentioned assay system based on the detachment of tissue-cultured BHK cells, we have recently found that a soluble toxic substance, which was not a product of amoebic lysis, was slowly released by intact trophozoites that were incubated in maintenance media (Gillin & Diamond 1980) (Fig. 7). The secretion of the cytotoxic activity was inhibited when cytochalasin B was added to the incubation mixtures (C. Feingold et al, unpublished observations). The cytopathic effect on monolayers of mammalian tissue-cultured cells was inhibited by several serum factors including fetuin (Mattern et al 1980, Kobiler et al 1981) α-macroglobulin and α-1-

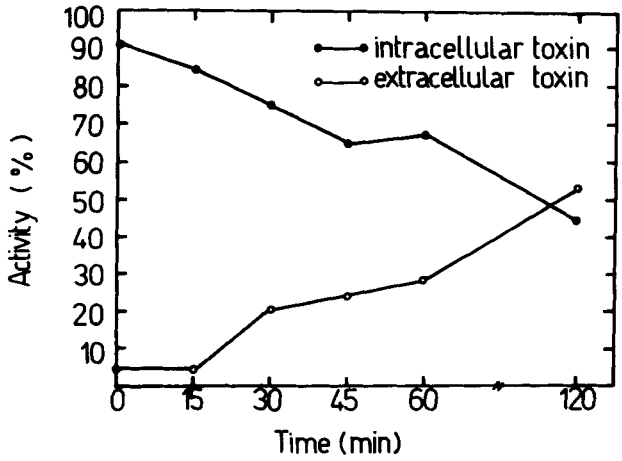

FIG. 7. Time-dependent release of cytopathic activity, by trophozoites of strain HM1 : IMSS, on incubation at 37 °C in saline that contained minimal media without serum. After incubation, the trophozoites were sedimented and the cytopathic activity released into the medium, as well as that remaining in the cells, was determined by their effect on baby hamster kidney cells.

TABLE 5 Inhibitors of the cytopathic activity of cell-free extracts of *E. histolytica*

Inhibitors	*Concentration added (mg/ml)*	*Destruction of tissue-culture monolayer (%)*
None	–	>95
Fetuin	2	17
Fetuin	25	<5
Asialofetuin	1	72
Thyroglobulin	2.5	28
Mucin	2.5	<5
Sialic acid	10	15
Sialic acid	4	35
Concanavalin A	1	40
Wheat-germ agglutinin	1	20
Soybean agglutinin	1	>95
Peanut agglutinin	1	>95

The tissue-cultured cells (baby hamster kidney) were preincubated with the lectins before being exposed to the cell-free extract (from 10^6 amoebae/well) The glycoproteins were added to the amoebic extract. The cell-free extract was obtained by freeze–thaw disruption of trophozoites of *E. histolytica* strain HM1 : IMSS.

antiprotease (Lushbaugh et al 1981). We have recently found that the cytopathic effect could also be inhibited by sialic acid-containing compounds (Table 5) and by precoating of the cells with wheat-germ agglutinin (1 mg/ml)

but not by soybean or peanut lectin, which indicates that sialic acid may act as a recognition site on the mammalian cell surface.

Since homogenates of non-pathogenic strains such as HK-9 and NIH : 200 had very small amounts of the toxic substance (Bos 1979), it was interesting to see whether these cell-free cytopathic activities would increase after pre-interaction of the HK-9 trophozoites with bacteria. Interaction between *E. histolytica* strains HK-9 or HM1 : IMSS and *E. coli* 7343 or 055 at a ratio of 1 : 1000, for 1 h before homogenization, produced a remarkable increase in the cytopathic activity of the amoebae (Fig. 8). The increase was greater for

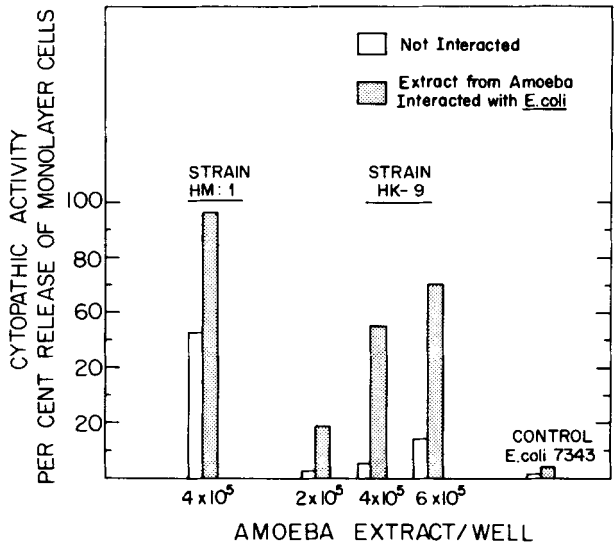

FIG. 8. Cytopathic activity of homogenates of *E. histolytica*, strains HK-9 and HM1 : IMSS, on baby hamster kidney cells before and after interaction with *E. coli* 7343.

strain HK-9 than for strain HM1 : IMSS. No increase was observed when homogenates of amoebae and bacteria were used or when sonicates of both bacteria and amoebae were used. Neither was there an increase in virulence when we used bacteria that did not adhere to the amoebae, such as *Shigella* (see above), or heat-inactivated or glutaraldehyde-fixed bacteria (Table 4).

Concluding remarks

One of the fundamental aspects of studying amoebiasis is to elucidate the factors that determine virulence of the parasite and its conversion sometimes

from a harmless commensal to an aggressive invader. It is generally accepted that a balanced host–parasite equilibrium facilitates the commensal relationship and that alterations in this balance may convert it to a virulent state. Disruption of the balance may be due to changes in either the host or parasite. Among a variety of possible host factors, consideration has been given to geographical location, race, sex, age, nutritional and immunological condition, diet, local climate and previous history of medication. Very little is known, however, about how such factors specifically alter the host. The parasite can also be affected apparently by many factors. Numerous strains with different pathogenicities have been isolated, and studies in several laboratories have begun to shed light on these differences as well as on the conditions that modify the degree of virulence in different strains.

Our results clearly indicate that specific mechanisms of carbohydrate recognition are critical for the interaction of trophozoites with both mammalian and bacterial cells. Moreover, our studies support the notion that association of axenically grown trophozoites with certain types of bacteria augments their virulence. The molecular mechanism behind the increased amoebic virulence is not yet clear. Transfer of bacterial genetic information is not likely to be the cause since the augmentation is quite rapid and plasmids are apparently not essential. Furthermore, inhibitors of protein or DNA-synthesis do not inhibit the augmentation. More appealing is the idea that bacterial ingestion supplies the trophozoite with a key nutrient ingredient (for example, iron) or an enzymic activity that liberates a cryptic virulent potential. It is tempting to speculate that most trophozoites have the potential for virulence and that certain conditions may trigger its expression.

The wider implications about the role of bacteria in the establishment of *E. histolytica* colonies and in the subsequent invasion of the colonic mucosa are far from being understood. On the one hand, certain bacteria that are not recognized or taken up by the amoebae may hinder the direct contact and attachment of the trophozoite to the apical surface of the intestinal epithelial cell (Martinez-Palomo 1982). On the other hand, bacterial strains that readily adhere to and are ingested by the amoebae may serve both as focal points of attachment to the intestinal mucosa and as triggers for enhanced trophozoite virulence. The nature of the intestinal microbial flora may thus have considerable importance for the pathogenicity of the amoebae and for the aetiology of the disease.

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DISCUSSION

Piras: Are you not worried about fixing the mammalian tissue-cultured cells with glutaraldehyde, which can make some surface components unavailable for other interactions?

Mirelman: I agree that glutaraldehyde fixation is not an ideal method, but we find so many difficulties in trying to dissociate the step of amoebic adherence from the step of tissue-cultured cell destruction that we have to resort to this type of technique. Using various controls we found that the mechanism of amoebic adherence that was sensitive to carbohydrates was unchanged when we used as target cells the glutaraldehyde-fixed monolayers.

Piras: What happens when you use low temperatures?

Mirelman: Amoebae will 'round up' at low temperatures or in the presence of cytochalasin B, and under these conditions they will not bind to mammalian

cells or inert surfaces. The amoebae have to be at an optimum temperature to form the filaments and filopodia needed for adherence.

Nussenzweig: You mentioned that you can inhibit the first phase of interaction by addition of sugars. But once the amoebae have adhered to the cells, can you inhibit the second phase i.e. pathogenicity?

Mirelman: It is almost impossible to reverse the first stage. As I mentioned, if α-methylmannoside is added after amoebae have adhered to bacteria, one cannot remove the bacteria from the amoebae, except at low temperature, in which case ingestion of the bacteria does not occur. It seems, therefore, that the initial recognition is mediated by a carbohydrate recognition mechanism but this step is rapidly followed by a stronger but, as yet, unclear attachment mechanism.

Chang: Have you followed the fate of the bacteria within the amoebae? Protozoa very often ingest intracellular bacteria. In some cases (e.g. in the work of Jeon & Jeon 1976) one can even establish intracellular symbiosis by feeding *Amoeba proteus* with bacteria. Another point is in relation to the *kappa* particle in *Paramecium* (Preer et al 1974). There appears to be some kind of bacteriophage which helps the bacteria or kappa particles to release a toxin that kills sensitive *Paramecium* without the particles. Does this connect with your work?

Mirelman: This is a very interesting question. A relevant example comes from recent findings on *Legionella* bacteria. Their natural reservoir appears to be in water-borne amoebae which hold the *Legionella* and give rise to the associated bacterial disease when the amoebae are inhaled in damp or closed quarters (air-conditioning water reservoirs). Ten minutes after the bacteria are inside the *Entamoeba histolytica* trophozoite, they are extremely sensitive to detergents, unlike most bacteria, and their outer membrane ceases to function as a protective barrier so they are dissolved. After two hours, thin sections of amoeba trophozoites under electron microscopy show that the bacteria are partially digested. Most bacteria under these conditions have no survival potential inside the amoebae. The possibility of using a potentially toxic substance from the bacteria to enhance the virulence of the amoebae has not escaped our notice. In Canada, Dr De Voe is finding that when *Neisseria meningitidis* (De Voe 1976) are ingested by macrophages, the macrophages cannot digest the endotoxin of the *Neisseria*. This endotoxin is released by the macrophages and can cause the toxic symptoms just as if it were the bacterium itself. It is possible that the amoebae cannot digest some bacterial toxins, e.g. lipopolysaccharide, for which no degrading enzymes have been found. We often use bacteria that are completely non-toxic—i.e. the K-12 derivatives that have no endotoxins and no exotoxins, and yet are capable of augmenting amoebic virulence. However, it is possible that in some cases the effects may be compounded, and the bacteria may also contribute some intrinsic toxic effect.