VIRULENCE OF *ENTAMOEBA HISTOLYTICA* TROPHOZOITES

Effects of Bacteria, Microaerobic Conditions, and Metronidazole

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Our knowledge about the factors that determine the virulence of the intestinal parasite *Entamoeba histolytica* and its conversion sometimes from a harmless commensal to an aggressive invader is rather limited (1). The development by Diamond (2, 3) of in vitro axenic cultivation methods for *E. histolytica* has enabled the study of many aspects of amebic virulence in the absence of other microorganisms with which the trophozoites are usually associated. Axenically cultured trophozoites of *E. histolytica* isolated from a number of amebiasis patients are known to vary in their degree of virulence when tested by several experimental criteria such as: (*a*) formation of hepatic abscesses in hamsters (4–6); (*b*) destruction of tissue-cultured mammalian cells (7, 8); (*c*) erythrophagocytosis (9, 10); and (*d*) presence of soluble enterotoxic substances (11–13).

Several investigators have indicated that amebic virulence decreased upon prolonged axenic cultivation (6, 14, 15), and increased when trophozoites were passaged through hamster liver (4, 16, 17) or reassociated with bacteria (18–20). Wittner and Rosenbaum (20) found that the ability of axenically grown trophozoites to cause hepatic lesions in hamsters was markedly enhanced after reassociation with bacteria. The aim of this work was to try to elucidate the bacterial contribution that leads to the augmentation of amebic virulence. Our present findings on the effects of various bacteria and conditions and their influence on amebic virulence cast further light on this complex process.

Methods and Materials

Entamoeba histolytica. The *E. histolytica* strains used throughout these studies were HK-9, HM-1:IMSS, and 200:NIH, which were obtained from Dr. L. Diamond, National Institutes of Health. Axenic cultivation of the trophozoites was as described by Diamond (3). Trophozoites were harvested during log phase growth after 48–72 h subculture by chilling the cultures in ice water (5 min) and sedimentation (300 *g*, 10 min).

Bacterial Strains. Escherichia coli serotype 055 and the *E. coli* clinical isolate 7343 (serotype 0115) as well as *Serratia marcescens*, *Shigella flexneri* 1b, and *Salmonella greenside* 050 were obtained from Dr. G. Altmann, Sheba Hospital, Israel and were grown as described (21–24). *E. coli* 09:K29$^-$ and *E. coli* 09:K29$^+$H$^+$ were obtained from Dr. M. Horwitz, The Rockefeller Univerity, New York. The *E. coli* strains (CR 63 and CA 274), known to be devoid of any plasmids, were obtained from Dr. S. Sarid, Weizmann Institute.

This work was supported by a grant from the Rockefeller Foundation. Address correspondence to D. Mirelman.

A catalase-deficient mutant of *E. coli* CAT 4, which contains <10% of wild-type catalase, was obtained from Dr. E. Yagil, University of Tel Aviv. *Shigella dysenteriae* type I (strain 3818 0) was obtained from Dr. G. Keusch, Tufts University, Boston. The presence of catalase activity in the bacterial strains was determined by phenol red and horseradish peroxidase as described (25). Cultures of *E. coli* 0115 (7343) or *E. coli* 055 were also grown in the presence of amikacin (10 μg/ml), cephalexin (8 μg/ml; a gift from Eli Lilly & Co., Indianapolis, IN), nalidixic acid (50 μg/ml; Sigma Chemical Co., St. Louis, MO), and bicyclomycin (200 μg/ml; a gift from Dr. F. Kradolfer, Ciba-Geigy, Switzerland) for 60 min before their harvest. Under these conditions, amikacin and nalidixic acid almost totally inhibited bacterial mass increase, as determined by the lack of increase of optical density of the cultures, whereas in cephalexin- or bicyclomycin-treated cultures, cell mass increased as in controls. Microscopic observation of the amikacin-treated bacteria did not reveal any morphological alteration. However, bacteria grown in the presence of cephalexin, bicyclomycin, and nalidixic acid grew as nondivided filamentous cells (26).

E. coli 055 were also grown in the presence of methyl viologen (Paraquat, 0.1–1.0 mM; Sigma Chemical Co.) for 2 h, a condition known to induce production of considerably higher (sixfold) levels of superoxide dismutase (27). For some experiments, samples of *E. coli* cells were sterilized by lethal γ radiation with a cobalt source (500,000 rad). No colony-forming units were detected after such treatment upon plating of bacteria on nutrient agar. Antibodies against *S. flexneri* 1b were prepared after the injection of fixed bacteria to the footpads of rabbits, as previously described (24). Opsonization of *S. flexneri* 1b cells with their specific antibodies was as reported (24).

Tissue-cultured Monolayers. Monolayers of baby hamster kidney (BHK)[1] cultured cells were grown until confluency in 24-well plates (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal calf serum for 60–72 h.

Quantitative Assay for the Determination of Tissue Culture Destruction. Interaction between trophozoites and tissue-cultured cells was as follows. In routine experiments, trophozoites (2×10^5/ml suspended in DME medium) were added to wells containing a confluent monolayer of BHK cells (2×10^5/well) that had been washed with DME to remove traces of fetal calf serum. Incubation with occasional mixing was usually carried out for 1 h at 37°C in a CO_2-containing incubator. The reaction was stopped by cooling the tissue culture plate (10 min, 4°C) to release adhered trophozoites and the plates were carefully washed twice with cold saline, fixed with 4% formaldehyde for 10 min, and finally washed twice with saline. The cells remaining on the tissue culture plate were stained with methylene blue (0.1% in borate buffer, 0.1 M, pH 8.7) for 10 min. The excess stain was washed repeatedly (three times) with 0.01 M borate buffer and the incorporated dye was extracted from the stained cells by HCl (1 ml, 0.1 M) at 37°C for 30 min. The amount of dye extracted was proportional to the amount of intact cells that remained attached to the tissue culture well (28). The amount of dye extracted from monolayers of tissue-cultured cells that had not interacted with trophozoites served as controls (0% destruction). The intensity of color was measured in a spectrophotometer (at 660 nm) after appropriate dilution with HCl (0.1 M). Experiments were carried out in duplicate, repeated at least twice, and standard error determined. To decide the exact time at which to terminate an experiment, the progress in tissue culture damage during the incubation with trophozoites was also monitored by periodic observation under an inverted microscope.

Interaction Between Bacteria and Trophozoites. Freshly harvested and washed trophozoites (see above) were incubated with log phase–grown bacteria at a ratio of 1:1,000 (2×10^5 ameba with 2×10^8 bacteria in 1 ml DME) for 15 min at 37°C. After this incubation the whole mixture was applied to the tissue culture monolayers and the incubation (1 h) was carried out as described above. In some experiments, the ameba/bacteria ratios as well as the bacterial growth conditions were modified as indicated.

Interactions Under Microaerobic Conditions. The microaerobic conditions used for some of the experiments were as follows. Trophozoites of *E. histolytica* were harvested as

[1] *Abbreviations used in this paper:* α-MM, α-methyl-D-mannoside; BHK, baby hamster kidney; DME, Dulbecco's modified Eagle's medium.

described above and maintained during their preparation under a nitrogen-containing atmosphere. The incubation of trophozoites with and without bacteria (15 min), as well as the interaction of trophozoites with the monolayers in the tissue culture plates were done in an anaerobic jar containing microaerobic conditions generated by a gas-producing kit (Oxoid, Hampshire, England) for *Campylobacter*. The jar was prewarmed at 37°C under anaerobic conditions for 30 min before the beginning of the experiment and the incubation of the ameba and tissue culture plates was for 60 min. After this period the plates were removed from the jar and the mammalian cells remaining on the tissue culture wells determined as described above.

Attachment of Bacteria to E. histolytica Trophozoites. The attachment of bacteria by *E. histolytica* trophozoites was determined as previously reported (22, 23). In principle, bacteria grown overnight with [^{14}C]glucose (The Radiochemical Centre, Amersham, England) were interacted for various periods with trophozoites, after which the nonattached bacteria were removed by a discontinuous Percoll gradient and the adhered bacteria determined by counting in a scintillation counter (22).

Uptake of Radiolabeled Metronidazole by Trophozoites of E. histolytica. The uptake of [^{14}C]metronidazole (a gift of G. D. Searle & Co., Skokie, IL) by trophozoites of *E. histolytica* HM-1:IMSS was determined as follows. Reaction mixtures containing 10^6/ml trophozoites were suspended in saline solution (1 ml) together with or without bacteria (10^9) at 37°C. [^{14}C]metronidazole at 25 μg/ml (8,000 cpm/μg) was added and incubations were carried out for 30 min at 37°C. The reaction was stopped by the addition of cold (4°C) saline (5 ml) and the rapid sedimentation of the trophozoites at 300 *g* for 5 min. The trophozoites were washed again with cold saline and then separated from nonadhered bacteria by a Percoll gradient as described before (22). The band containing the trophozoites (at 30–50% Percoll) was removed and the radioactivity incorporated counted by scintillation with a Triton X-100–based scintillation cocktail. The uptake of [^{14}C]metronidazole into bacteria alone was done by counting the sedimented bacteria (10,000 *g*, 15 min). Controls which proved that [^{14}C]metronidazole did not concentrate in Percoll gradients were also done.

Galactose and *N*-acetylgalactosamine were purchased from Pfanstiehl Laboratories, Inc., Waukegan, IL. Lactose, and α-methyl-D-mannoside (α-MM), beef liver catalase, and superoxide dismutase were from Sigma Chemical Co. Metronidazole (Flagyl) in solution (5 mg/ml) was from Specia, Paris, France. Emetine was from Eli Lilly & Co. and tetracycline from Lederle Laboratories, Wayne, NJ. Hydrogen peroxide was from Fluka A.G., Basel, Switzerland. Horseradish peroxidase type I was from Bio Yeda, Rehovoth, Israel.

Results

Determination of Amebic Virulence. Incubation of *E. histolytica* trophozoites with monolayers of tissue-cultured cells has been shown (1, 7, 8) to cause the gradual release and ingestion of mammalian cells by the parasite. Based on these observations, the virulence of *E. histolytica* trophozoites was determined with an assay that semiquantifies the rate of destruction of tissue-cultured monolayers by staining with methylene blue the mammalian cells that remain after incubation with trophozoites and extraction of the retained dye. No damage to the monolayer was observed during the first 30 min of incubation or when incubations were done in the cold (4°C). Damage to the tissue culture was a function of the strain and amount of *E. histolytica* trophozoites added (Fig. 1). At the ratio of one trophozoite (strain HM-1:IMSS) per mammalian cell (2×10^5), the average incubation time for the destruction of 50% of the tissue-cultured monolayer was ~60 min. At lower ratios of trophozoite to target cell, lysis of monolayers was considerably slower (Fig. 1).

Inhibition of BHK monolayer destruction by the ameba trophozoites was observed, as previously shown (8, 30, 32), in the presence of a number of

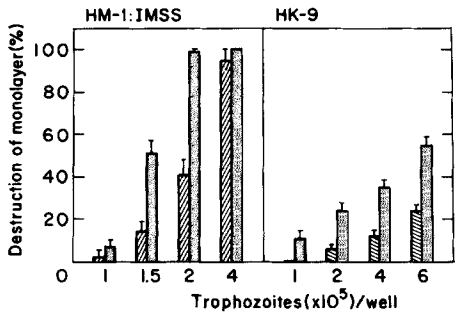

FIGURE 1. Destruction of tissue-cultured monolayers of BHK cells (average 2×10^5/well) by increasing numbers of trophozoites of *E. histolytica* strains HM-1:IMMS (*left*) or HK-9 (*right*) in the absence ▨ or presence □ of added bacteria (*E. coli* 055). Trophozoites were preincubated with bacteria for 15 min at a ratio of 1,000:1 (bacteria/trophozoite) in 0.5 ml DME, after which the incubation mixture was added to the monolayers and further incubated for 60 min at 37°C in a CO_2 incubator. After the incubation, the tissue culture plates were put on an ice-water bath for 10 min and repeatedly washed with cold saline to remove attached trophozoites. The mammalian cells that remained in the wells after the incubations were determined by staining with methylene blue as described in Materials and Methods.

carbohydrates that inhibit the ameba recognition and adherence mechanisms (8, 30). The best inhibitors were galactose, lactose, and *N*-acetylgalactosamine, which at 0.5% (final concentration) inhibited (>90%) the destruction of the tissue culture monolayer.

Effects of Bacteria on Amebic Virulence. Preincubation of ameba trophozoites of strain HK-9 (2×10^5) with increasing amounts of *E. coli* 0115 (7343) cells, a bacterial strain that has a surface lectin specific for mannose residues (21), gradually increased the rate of monolayer destruction. Maximal destruction rates were observed when the ratio between trophozoites and bacteria was 1:1,000 (Fig. 2), although at a ratio of 1:10, a small increase in the rate of destruction above that of trophozoites without bacteria could be seen. Bacteria alone had no effect on the tissue culture cells (Fig. 2). The addition of α-MM (1%) to the bacteria/ameba mixture (at a 1:100 ratio) to provide conditions that inhibit (>80%) of the attachment of such bacteria to the amebae (22), prevented most of the increase in amebic virulence (Fig. 2). Other mannose-binding bacteria such as *S. marcescens* (31) as well as *E. coli* strains known not to contain plasmids or cured from plasmids by acridine orange treatment (strains CR63 and CA274) also augmented amebic virulence (Table I). Stimulation of amebic virulence was also observed with an *E. coli* mutant almost devoid of catalase (Cat 4), as well as with the catalase-negative *S. dysenteriae* I (Tables I and V).

Increased amebic virulence was observed after reassociation not only with bacterial strains that possess mannose-binding lectins (31), but also with other types of bacteria that were found to attach to the amebae by virtue of having cell surface carbohydrates that serve as binding sites for the ameba lectin (8, 23, 32). The strains that attached to the ameba by such a mechanism and induced augmentation of virulence were *E. coli* serotype 055, *S. greenside* 050, and *S. dysenteriae* type I (Table I). In these cases, galactose or lactose (5 mg/ml) inhibited both the interaction of the trophozoite with the mammalian cell (8) and its ability to attach *E. coli* 055 and *S. greenside* 050 cells (23).

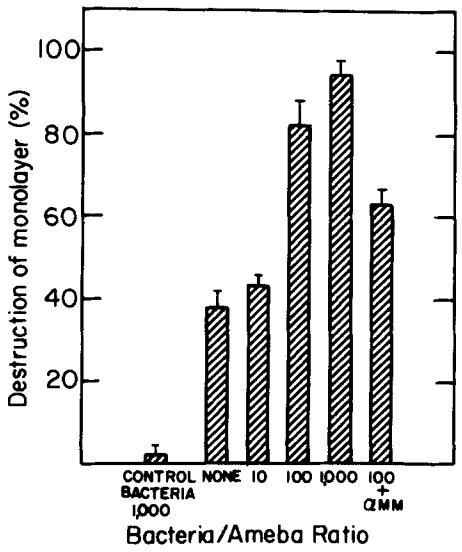

FIGURE 2. Effect of associated bacteria on the rate of destruction of tissue culture monolayers by trophozoites of *E. histolytica*. Varying amounts of *E. coli* 0115 cells were preincubated with *E. histolytica* strain HM-1:IMSS (2×10^5 in 1 ml) for 15 min, after which the incubation mixture was added to the monolayers of BHK cells at a trophozoites/BHK cell ratio of 1:1 and further incubated for 60 min. Determination of mammalian cells remaining in the wells after the incubation was as described in Materials and Methods. Controls containing only bacteria (2×10^8) were also incubated. To one of the experiments (at a ratio of 100 bacteria per ameba), α-MM (0.5% final concentration) was added at the beginning of the incubation to inhibit the attachment of the *E. coli* cells to the trophozoites (22).

Association of amebae with *S. flexneri* serotype 1b, *S. aureus* H, or with bacterial strains such as *E. coli* 09:K29$^+$H$^+$ or *E. coli* 09:K29$^-$H$^-$, which were known not to become attached or ingested by the trophozoites (23, 32), did not stimulate their virulence (Table I). A slight augmentation of virulence was observed upon association of amebae with opsonin-coated *S. flexneri* or *S. aureus* (Table I), conditions that enabled attachment and ingestion by the trophozoites (22).

Enhancement of amebic virulence after association with bacteria was detected in several axenically cultured *E. histolytica* strains (HK-9, HM-1:IMSS, and 200:NIH). Some variability existed, however, in the rates of tissue culture destruction that each ameba strain displayed before its association with bacteria. Trophozoites of strains HM-1:IMSS were usually the most virulent among the three strains tested and their rates of tissue culture destruction were faster. A higher number of trophozoites of strain HK-9 per mammalian cell were needed to attain lysis rates similar to those observed for strain HM-1:IMSS (Fig. 1).

Pretreatments of Bacteria. For amebic virulence to be stimulated, certain conditions were required from the bacteria (*E. coli* 055 or *E. coli* 0115). Maximal augmentation of virulence was observed when preincubations between trophozoites (2×10^5/ml) and bacteria (at a ratio of 1:1,000) were between 15 min and 1 h. No difference was found when using log or stationary growth phase bacteria or with bacteria grown in rich or minimal media. On the other hand, heat-inactivated, glutaraldehyde-fixed, or sonicated bacteria did not stimulate virulence. Neither did the bacterial cell envelope or cytosol fractions obtained after

TABLE I

Destruction of Tissue Culture Monolayers by Trophozoites of E. histolytica Strain HK-9 After Interaction with Different Bacteria

Bacterial strain associated with trophozoites	Pretreatment of bacteria	Specificity of adherence to trophozoite*	Tissue culture destruction (percent damage above ameba alone)‡
None			0
E. coli 055	—	Galactose	68 ± 5
Salmonella greenside 050	—	Galactose	62 ± 3
Shigella dysenteriae 1	—	Galactose	43 ± 4
E. coli 0115 (7343)	—	Mannose	54 ± 3
E. coli K_{12} CR63§	—	Mannose	52 ± 2
E. coli K_{12} CA 274§	—	Mannose	40 ± 5
E. coli 09:K^+ 29H^+	—	None	<7
E. coli 09:K^- 29H^-	—	None	<7
Shigella flexneri 1b	—	None	<7
Shigella flexneri 1b	Opsonized	Galactose	10 ± 3
Staphylococcus aureus H	—	None	<7
Staphylococcus aureus H	Opsonized	Galactose	7 ± 3

* Carbohydrate recognition specificity between bacteria and trophozoite (22, 23).

‡ Results express damage to tissue cultures above a control in which ameba trophozoites without bacteria were interacted with the tissue culture monolayer. Tissue culture destruction by trophozoites alone was 18%. Destruction was determined from the amount of cells remaining after interaction. For experimental details see Materials and Methods.

§ *E. coli* strains that do not contain plasmids.

mechanical disruption of bacteria in a French press (Table II). Stimulation of amebic virulence was observed, however, with bacteria that had been exposed to a lethal dose of γ radiation from a cobalt source (500,000 rad). Bacteria (either *E. coli* serotype 0115 or 055) grown for three generations (60 min before harvest) with cell wall synthesis inhibitors such as cephalexin (10 μg/ml) or bicyclomycin (200 μg/ml), which induced morphological alterations (filamentous forms), were capable of adhering to the trophozoites and stimulating their virulence (Table II). Pretreatments of bacteria with protein synthesis inhibitors such as amikacin (100 μg/ml), which markedly affected bacterial growth but did not cause lysis, strongly decreased their ability to stimulate amebic virulence (Table II). In addition, nalidixic acid–grown bacteria had a decreased ability to stimulate virulence. Virulence of trophozoites, as well as its stimulation by bacteria, was not affected, however, when tetracycline (50 μg/ml), amikacin (100 μg/ml), or nalidixic acid (50 μg/ml) were added to incubation mixtures containing trophozoites, together with normally grown *E. coli* 055 cells on tissue-cultured monolayers. On the other hand, emetine, the well-known antiamebal agent (29), almost totally inhibited amebic virulence even in the absence of added bacteria. In the presence of this drug (20 μg/ml), trophozoites attached comparable

TABLE II

Pretreatments of Bacteria and their Effects on their Ability to Stimulate Amoebic Virulence

Pretreatment of bacteria	Additions to the tissue-cultured cells*	Relative rate of destruction of tissue-cultured monolayers‡
Untreated	—	1.00 ± 0.05
Sonicated	—	0.17 ± 0.02
Mechanically disrupted (French press)		
Envelope fraction	—	<0.02
Cytoplasmic fraction	—	0.02 ± 0.05
Glutaraldehyde fixation	—	<0.02
Heat (121°C, 15 min)	—	<0.02
γ Irradiation, 20,000 rad	—	1.04 ± 0.10
100,000 rad	—	0.84 ± 0.07
500,000 rad	—	0.64 ± 0.10
Cephalexin (10 μg/ml, 90 min)	—	0.88 ± 0.03
—	Cephalexin, 10 μg/ml	0.88 ± 0.03
Bicyclomycin (200 μg/ml, 90 min)	—	1.14 ± 0.03
—	Bicyclomycin, 200 μg/ml	1.26 ± 0.04
Amikacin (100 μg/ml, 60 min)	—	0.32 ± 0.05
—	Amikacin, 100 μg/ml	0.99 ± 0.05
Nalidixic acid (50 μg/ml, 60 min)	—	0.57 ± 0.06
	Nalidixic acid, 50 μg/ml	1.12 ± 0.10

* The addition of antibiotics to the monolayers was done at the beginning of the incubation between ameba and the tissue-cultured cells.

‡ Results are based on damage to tissue-cultured monolayers after incubation (60 min) with *E. histolytica* strain HK-9 trophozoites. Destruction of tissue culture monolayers under these conditions was 12 ± 3%, whereas the level of destruction after association with *E. coli* 0115 (at a ratio of 1:1,000) was 64 ± 3. This level of monolayer destruction was taken as 1.00, and the destruction in the absence of bacteria as 0.00, augmentation. The results of all other experiments were compared with these values.

numbers of bacteria (see Table IV), but did not destroy tissue culture monolayers (<10%).

Effect of Metronidazole. One of the compounds that had a marked specific effect on the augmentation of amebic virulence by bacteria was metronidazole (50 μg/ml). Its addition to incubation mixtures containing trophozoites and normally growing *E. coli* 055 cells lowered the virulence to a level below that of trophozoites alone (Fig. 3). On the other hand, controls showed that at least for the duration of the experiment (60 min), metronidazole had no marked effect on the basal level of virulence of the trophozoite (in the absence of bacteria). Growth of *E. coli* 055 was not affected by metronidazole, and bacteria grown with metronidazole were capable of augmenting virulence. Furthermore, the presence of metronidazole did not inhibit the adherence of *E. coli* 055 (Table III) and similar numbers of bacteria were found attached after 30 min incubation.

The uptake of [^{14}C]metronidazole by trophozoites of *E. histolytica* (strain HM-1:IMSS) was markedly enhanced in the presence of *E. coli* strains 055 or 0115. Only live or γ-irradiated bacteria stimulated the uptake of [^{14}C]metronidazole

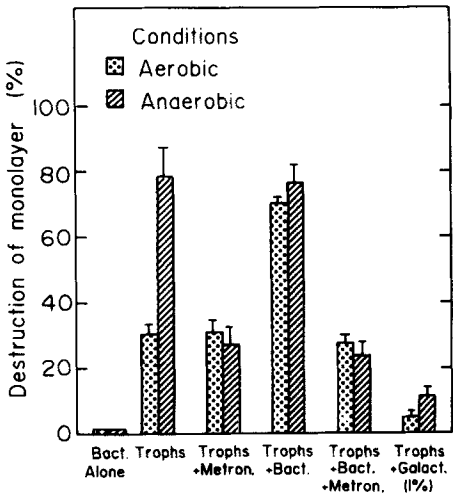

FIGURE 3. Effect of microaerobic conditions, bacteria, and metronidazole on the rate of destruction of tissue-cultured monolayers by trophozoites of *E. histolytica* strain HM-1:IMMS. Incubations of trophozoites with tissue culture monolayers under anaerobic conditions were done in a jar as described in Materials and Methods. Metronidazole (final concentration, 50 μg/ml) was added to trophozoites at the beginning of the incubation. The amount of mammalian cells remaining in the wells after the incubation was determined as described in Materials and Methods. The inhibitory effect of galactose (10 mg/ml, final concentration) on the destruction of monolayers by the trophozoites under both conditions was also determined.

TABLE III

Attachment of Bacteria (E. coli 055) by Trophozoites of E. histolytica Under Various Conditions

Conditions and additions	Adherence (bacteria/ trophozoite)
Aerobic	68 ± 5
Anaerobic	56 ± 4
Metronidazole (50 μg/ml)	67 ± 6
H_2O_2 (200 μM)	55 ± 4
Emetine (10 μg/ml)	60 ± 6
Cephalexin (50 μg/ml)	68 ± 5

Trophozoites (strain HM-1:IMSS, 10^6) were suspended together with ^{14}C-radiolabeled *E. coli* 055 (10^9) in 1 ml and incubated in saline at 37°C for 30 min. After the incubation trophozoites were separated from nonattached bacteria in a discontinuous Percoll gradient and the number of bacterial cells attached to the trophozoites was determined as described (22, 23).

by the trophozoites. Heat-inactivated bacteria had no such effect. Bacteria alone did not incorporate the drug (Table IV).

Effect of Microaerobic Conditions. As shown in Fig. 3, the virulence of amebae, as determined by the rate of destruction of tissue culture monolayers, was clearly augmented when the interactions were carried out in an anaerobic jar with a gas-generating kit for microaerobic conditions (5% O_2, 10% CO_2). Under these conditions the rate of tissue culture destruction by trophozoites was higher than under atmospheric conditions, and the levels of augmentation due to association

TABLE IV
Uptake of [^{14}C]Metronidazole by Trophozoites of E. histolytica in the Presence or Absence of Bacteria

Experiment	Uptake of metronidazole (pmol/10^6 trophozoites)
Trophozoites	6.8
Trophozoites + *E. coli* 055	15.4
E. coli 055	0.65
Troph. + *E. coli* 055 (heat denatured)	8.9
Troph. + *E. coli* 055 (x ray irradiated)	14.1
Troph. + *E. coli* 0115	13.8
Troph. + *E. coli* 0115 (heat denatured)	8.6

Concentration of [^{14}C]metronidazole was 25 μg/ml (sp act, 8,000 cpm/μg). Incubation of trophozoites (10^6/ml) was for 30 min at 37°C. Bacteria were added at a ratio of 1,000:1. After the incubation, trophozoites were separated from bacteria by Percoll gradients as described (22).

with bacteria were lower (Fig. 3). Metronidazole inhibited the higher rates of tissue culture destruction obtained under anaerobic conditions as well as the increase obtained due to interaction of bacteria (Fig. 3). Galactose (1%), as previously shown (8), inhibited both the aerobic and anaerobic destruction of tissue-cultured monolayers (Fig. 3).

Effects of Hydrogen Peroxide. *E. histolytica* trophozoites are known to be devoid of catalase (39) and are very sensitive to hydrogen peroxide (33). In the presence of hydrogen peroxide (>100 μM), trophozoites exerted no damage to tissue-cultured cells (Fig. 4). Their interaction with bacterial cells was, however, not significantly affected (Table III), and their virulence was stimulated even in the presence of relatively high concentrations of H_2O_2 (1 mM). Association with the catalase-deficient *S. dysenteriae* type I strain afforded less protection to the ameba, and augmentation of virulence was impaired at 200 μM H_2O_2. That intact bacteria, including *S. dysenteriae*, are capable of rapid destruction of hydrogen peroxide was revealed by the preincubation of bacteria with the hydrogen peroxide solution before its addition to the trophozoites. Solutions of 200 μM H_2O_2 incubated for <30 min with either *E. coli* 055 or *S. dysenteriae* I no longer had a significant inhibitory effect on the basal virulence of trophozoites (without bacteria).

Association of trophozoites with the *E. coli* CAT 4 mutant, which contains <10% of the catalase activity present in the wild-type *E. coli* K_{12}, stimulated amebic virulence even at low bacteria to trophozoites ratios (Table V). Exogenously added mammalian liver catalase had a small stimulatory effect on amebic virulence at high concentrations (1,000 U) (Table V), but no effect was observed with horseradish peroxidase (100 μg). *E. coli* 055 cells grown for 2 h in the presence of methyl viologen (1 mM) (27) did not significantly differ in their capacity to stimulate trophozoite virulence (Table VI). On the other hand, preincubation of trophozoites with methyl viologen (1 mM) or with superoxide dismutase (100 U) had a marked inhibitory effect on their ability to destroy tissue-cultured cells. The addition of bacteria abolished the inhibitory effects of

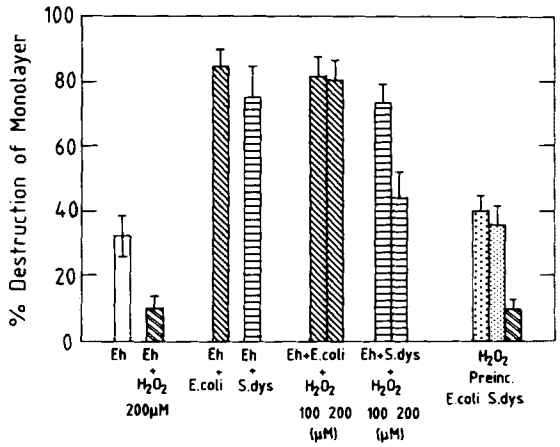

FIGURE 4. Effect of hydrogen peroxide and bacteria on the rate of destruction of tissue-cultured monolayers by trophozoites of *E. histolytica* (Eh) strain HM-1:IMSS. Addition of hydrogen peroxide (200 μM) markedly affected the ability of trophozoites to destroy tissue-cultured cells. Association with *E. coli* 055 (at a ratio of 1,000 bacteria per ameba) augmented their activity and protected the trophozoites from the toxic effect of H_2O_2. Association with the catalase-deficient *S. dysenteriae* was less protective, especially at high H_2O_2 concentrations (200 μM). Preincubation (30 min, 37°C) of H_2O_2 solutions (200 μM) with bacteria (2×10^8) or without bacteria before the addition of the hydrogen peroxide solution (without bacteria) to the trophozoites revealed that both *E. coli* 055 and *S. dysenteriae* I abolished the toxic effect, whereas H_2O_2 incubated without bacteria was still effective against trophozoite activity.

TABLE V

Destruction of Tissue Culture Monolayers by Trophozoites of E. histolytica Strain HM-1:IMSS and Effect of Catalase and the Catalase-deficient Mutant E. coli CAT 4

Bacteria added (ratio to trophozoites)	Additions to tissue-cultured cells	Percent destruction of monolayer
—	—	54 ± 3
E. coli K_{12} (200:1)	—	69 ± 5
E. coli K_{12} (1,000:1)	—	89 ± 7
E. coli CAT 4 (200:1)	—	60 ± 4
E. coli CAT 4 (1,000:1)	—	88 ± 6
—	Catalase (100 U)	55 ± 4
—	Catalase (1,000 U)	67 ± 6

Bacteria were preincubated with trophozoites (2×10^5 in 0.5 ml) for 15 min after which they were added to wells containing BHK cells and further incubated for 60 min. For experimental details see Materials and Methods.

methyl viologen or superoxide dismutase and restored amebic virulence (Table VI).

Discussion

The biochemical and molecular mechanisms that determine the virulence of *E. histolytica* trophozoites have yet to be elucidated. The degree of virulence of cultured *E. histolytica* trophozoites has been shown to vary according to the strain (4, 6, 9) and culture conditions (14, 15). Good correlation has been observed

TABLE VI

Destruction of Tissue-cultured Monolayers by Trophozoites of E. histolytica Strain HK-9: Effects of Methyl Viologen, Superoxide Dismutase, and Bacteria

Additions to trophozoites*	Bacteria added‡	Percent destruction of monolayer§
—	—	59 ± 5
—	*E. coli* 055	88 ± 6
—	*E. coli* 055 grown with methyl viologen	84 ± 8
Methyl viologen	—	36 ± 4
Superoxide dismutase	—	37 ± 3
Methyl viologen + superoxide dismutase	—	20 ± 2
Methyl viologen	*E. coli* 055	77 ± 4
Superoxide dismutase	*E. coli* 055	83 ± 5

* Trophozoites (4×10^5) were preincubated for 30 min with the various additions and then applied to wells containing BHK cells and further incubated for 60 min. Methyl viologen was added at 1 mM and superoxide dismutase at 100 U.

‡ Ratio of *E. coli* 055 to trophozoites was 1,000:1. In one experiment, *E. coli* 055 were grown for 2 h in the presence of methyl viologen (1 mM) before their addition to the trophozoites.

§ For experimental details see Materials and Methods.

between the various in vivo and in vitro experimental assays used for determining virulence (1). One of the better known pathogenic strains, HM-1:IMSS, very readily produces hepatic lesions in hamsters (5, 6), lyses cultured mammalian cells (8), and phagocytizes erythrocytes (10). It also has an active collagenase system (34, 35), as well as other soluble toxic substances that have not yet been fully characterized (11–13). Strains with attenuated pathogenicity, such as HK-9, have been repeatedly shown to have considerably less virulent properties in all these tests.

It is well established today that the adherence or attachment of a pathogen to a host cell is the initial step in pathogenicity (1, 22). In previous investigations (8, 30, 32), we and others have shown that specific carbohydrate recognition mechanisms mediate the interaction of trophozoites with both mammalian and bacterial cells. Low concentrations of galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine oligosaccharides almost totally inhibited the adherence, phagocytosis, and destruction of mammalian cells.

A number of clinical and experimental observations have indicated that interactions between ameba and bacteria are important for the expression of pathogenicity in *E. histolytica* (18–20, 32). The aim of this study was to investigate the bacterial role and contribution to amebic virulence. We have earlier shown that *E. histolytica* trophozoites are quite selective with regard to the types of bacteria they will interact with. Two lectin-mediated mechanisms that enable the initial attachment of bacteria to the trophozoite have been described (22, 23). Only bacteria that had mannose-binding lectins on their cell surface, or bacteria that

possessed galactose or *N*-acetylgalactosamine moieties as surface receptors for the ameba-lectin, became attached, ingested, and degraded by the trophozoite (22, 23). Preincubation of trophozoites, especially those from less pathogenic strains, with such types of bacteria, markedly enhanced their virulence, as evidenced by their increased ability to destroy tissue-cultured monolayers (Table I). Bacteria that were incapable of adhering to the trophozoites, either because they did not possess any of the above-mentioned lectin receptor–mediated attachment mechanisms (*E. coli* 09:K29H, *S. flexneri*, or *S. aureus*) or due to carbohydrate inhibition of their adherence, did not stimulate amebic virulence (Table I).

For the stimulation to be noted, trophozoites require the ingestion of a certain minimal number of bacteria (5–10 bacteria per ameba), and in the small volumes and high multiplicity ratios of amebae to bacteria used (1:1,000), this was shown to occur very rapidly (22, 23). Our results show that augmentation of amebic virulence could be achieved only with intact bacteria. Denatured (either by heat or by cross-linking agents), sonicated, or mechanically disrupted bacteria did not stimulate virulence. Bacteria that were lethally irradiated (500,000 rad) were, however, capable of increasing virulence, as were bacteria that were devoid of any plasmids (Table II). Growth of bacteria for short periods (60–90 min) with DNA or protein synthesis inhibitors (but not with cell wall synthesis inhibitors) before their interaction with the trophozoites markedly reduced their stimulative capabilities, suggesting that the ingested bacteria contribute to the trophozoites a proteinous ingredient or an enzymatic activity that rapidly activates a virulent response in the parasite.

Successful anaerobic cells must protect themselves from a number of highly reactive species of molecules and radicals generated directly or indirectly during the reduction of oxygen. Products toxic to anaerobic organisms are known to rapidly build up in culture media exposed to air and may actually arise from agents added to the medium to lower the oxidation-reduction potential (36, 37). These reactive products may damage cell components such as sulfhydryl and metaloprotein groups, cause a rise in the particular oxidation-reduction potential within the cell, and inhibit the electron transport system, thus consuming the cell's reducing power.

Although *E. histolytica* trophozoites are anaerobes that will slowly die in the presence of atmospheric conditions, they are known to have an affinity for oxygen and will consume some of it and metabolize it into water (38–40). Trophozoites have been shown to contain low levels of superoxide dismutase and glutathion peroxidase but virtually no catalase, and are very sensitive to hydrogen peroxide (33). To investigate the possible role that the above-mentioned toxic products may have on amebic virulence, we carried out experiments under microaerobic conditions (in anaerobic jars with gas-generating kits for *Campylobacters*, which are devoid of toxic hydrogen gas [41]), as well as in the presence of hydrogen peroxide. The results show that the virulence of trophozoites was considerably increased under microaerobic conditions and the rates obtained for mammalian cell destruction resembled those observed with associated bacteria in atmospheric ambient (Figs. 2 and 3). Little additional stimulation of virulence was observed upon association of trophozoites and bacteria under

anaerobic conditions. Association with bacteria, however, protected the trophozoites from the toxic effects of exogenously added hydrogen peroxide (Fig. 4). Moreover, our results with methyl viologen and superoxide dismutase show that the induction of oxidized molecules within the ameba inhibited their virulence, which could then be restored by the ingestion of bacteria (Table VI). On the other hand, agents that specifically consume the reducing power of the trophozoites, such as metronidazole, which is reduced by ferredoxin (42), drastically inhibited the amebic virulence both under anerobic conditions and in bacteria-stimulated trophozoites. Further evidence that considerably more metronidazole became reduced under conditions of amebic stimulation by bacteria can be deduced from the higher amounts of [^{14}C]metronidazole that were incorporated into the trophozoites (Table IV) (42).

Our results indicate that virulence of a given *E. histolytica* strain may depend to a considerable extent on the activity of their electron transport system, or the cell's reducing power. Both anaerobic conditions and the ingested bacteria apparently favor the lowering of the redox potential in the ameba cell and facilitate its electron transport system, the first by virtue of oxygen deprival (45), whereas ingested bacteria apparently function as broad range scavengers for oxidized molecules. Which of the various bacterial enzymatic systems, components, or products is responsible for this effect is not yet completely clear. Iron-binding proteins of the bacteria such as enterochelins (46) and cytochromes, as well as proteins rich in sulfhydril groups, may serve as electron donors and contribute to the reducing power of the trophozoite. Furthermore, although no conclusive evidence was obtained in this study for the direct contribution of bacterial catalase and superoxide dismutase to amebic virulence, it would be premature to discount their possible participation in protecting the trophozoites from highly toxic oxidized metabolites.

Very little is known about the conditions that determine amebic virulence in the human host. Pathogenic amebae isolated from patients with active amebiasis have been shown to display several isoenzyme electrophoretic patterns, which are quite distinct from those of amebae isolated from asymptomatic carriers (43, 44). Since the isoenzymes of axenically grown strains, analyzed until today, all show pathogenic patterns and since there are no axenic strains of nonpathogenic amebae, it is not yet possible to test whether virulence of the latter could be augmented under certain conditions.

Preliminary results recently obtained in our laboratory indicate that rates of erythrophagocytosis by axenically cultured trophozoites (strain HM-1:IMSS), which is another established criterion for pathogenicity (10), was also markedly increased under microaerobic or bacteria-associated conditions (E. Orozco, R. Bracha, and D. Mirelman, manuscript in preparation).

Our present findings may have important clinical implications. Our knowledge about factors that participate in determining the intestinal redox potential and the intracolonic gaseous tensions under different metabolic or environmental conditions is rather limited (47). Variations in feeding or diet habits, changes in the nature of the microbial flora, as well as conditions which lower oxygen tension such as found at high altitudes or due to poor blood circulation, may be factors that alter the microenvironment of the intestine and could in turn affect

amebic virulence. It would be interesting to know if a correlation exists between intestinal conditions that are stimulative for the ameba trophozoites and the incidence of virulent amebiasis.

Summary

The association of axenically grown trophozoites of *Entamoeba histolytica* strains HK-9 or HM-1:IMSS with various types of gram-negative bacteria for relatively short periods markedly increased their virulence, as evidenced by their ability to destroy monolayers of tissue-cultured cells. Interaction of trophozoites with bacteria that were heat inactivated, glutaraldehyde fixed, or disrupted by sonication, or bacteria treated with inhibitors of protein synthesis, did not augment amebic virulence. Lethally irradiated bacteria, however, retained their stimulative properties and trophozoites that ingested bacteria were protected from the toxic effects of added hydrogen peroxide. An increase in virulent properties of amebae was also found in experiments carried out under microaerobic conditions (5% O_2, 10% CO_2). The augmentation of amebic virulence due to association with bacteria was specifically blocked by metronidazole, but not by tetracycline or aminoglycosides, and the rate of metronidazole uptake in stimulated trophozoites was two to three times higher. The results obtained suggest that virulence of axenically grown *E. histolytica* trophozoites may depend to a considerable extent on the cell's reducing power. Both microaerobic conditions and the association with bacteria apparently stimulate the electron transport system of the ameba. Bacteria may function as broad range scavengers for oxidized molecules and metabolites through the contribution of enzymatic systems, components, or products.

We wish to thank Dr. Louis Diamond for his helpful advice in using the anaerobic kits for *Campylobacters* and Dr. Gideon Altmann for his bacterial strains and advice.

Received for publication 18 January 1984 and in revised form 9 April 1984.

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