

Growth Advantage and Enhanced Toxicity of *Escherichia coli* Adherent to Tissue Culture Cells due to Restricted Diffusion of Products Secreted by the Cells

Dina Zafriri,* Y. Oron,† Barry I. Eisenstein,‡ and Itzhak Ofek*

Departments of *Human Microbiology, †Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel; and ‡Microbiology and Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Abstract

This study was undertaken to examine whether *Escherichia coli* adherent to tissue cells gain advantages over nonadherent bacteria due to their proximity to the cells. We used tissue culture cells and isogenic derivatives of a proline auxotrophic strain of *E. coli* that were fimbriated (Fim⁺) or nonfimbriated (Fim⁻), and were heat-labile enterotoxin producing (Tox⁺) or toxin nonproducing (Tox⁻). We found that the Fim⁺ bacteria, which were capable of adhering to tissue culture cells, initiated growth much sooner than did nonadherent Fim⁻ bacteria; the adherent bacteria used tissue cell-derived proline, which was available at high concentrations only in the zone of bacterial adherence. Likewise, cyclic AMP secreted by adherent (Fim⁺) bacteria was maintained at high concentration on the tissue cell surfaces. As few as 2×10^5 adherent Fim⁺ Tox⁺ bacteria exert toxic activity upon Y1 adrenal cells, whereas toxin secreted in the medium by 6×10^6 Fim⁻ Tox⁺ bacteria was undetectable. The results suggest that the growth advantage and enhanced toxicity of adherent *E. coli* is due to restricted diffusion of products secreted by the tissue culture and bacterial cells, respectively.

Introduction

Bacterial adherence to mucosal cells is an important step in the infectious process for at least three reasons (1, 2). First, and probably the most important, is that bacterial attachment protects the bacteria from being swept away by the normal cleansing mechanisms operating on mucosal surfaces (e.g., urinary flow, peristalsis). Second, penetration of the mucosal barrier must somehow proceed by the adherence of the invading bacteria onto the cell surface. Finally, it has been postulated, without direct evidence, that secreted toxic products may reach their cell target more efficiently when secreted by adherent bacteria than when secreted by nonadherent bacteria (3).

Despite reasonable evidence, however, that bacterial adherence to mucosal tissues plays a major role in the infectious process, little information is available concerning the molecular mechanisms that confer an advantage to adherent, as compared with nonadherent, bacteria in their survival, proliferation, and

ability to cause tissue damage. In particular, little is presently known regarding the effect of bacterial adherence to tissues on either the ability of the growing bacteria to use nutrients liberated by the target tissue or the distribution of secreted bacterial products in the medium relative to those in the tissue-media interface. Consequently, this study was undertaken to examine these two secretion activities in a system, using tissue culture cells and various isogenic mutants of *Escherichia coli*. We found that there was both a growth advantage and an enhanced effect of heat-labile enterotoxin (LT)¹ in *E. coli* capable of adhering to tissue cells, possibly due to a high concentration, in crypts formed by the ruffle structures of the animal cells, of the respective secreted products.

Methods

Bacteria. The K-12 proline auxotrophic *E. coli* strains used in this study have been described elsewhere (4, 5). Briefly, they are the fimbriated (Fim⁺) strain CSH50, harboring either plasmid pBR322 or the pBR322-derived, LT⁺ plasmid, pΔ299 (kindly supplied by Dr. P. C. Tai of Harvard University), its nonfimbriated (Fim⁻) derivative, strain VL584, and a Fim⁺ Cya⁻ derivative strain VL391, which does not produce cyclic AMP (cAMP). Two additional proline auxotrophic strains of *E. coli* (AB 2659 and Met F3455) were also included (kindly supplied by Dr. E. Ron from Department of Microbiology, Tel-Aviv University). Bacteria were grown in trypticase soy broth at 37°C for 48 h.

Tissue culture cell line. The human intestinal epithelial cell-line CCL6 (ATCC collection) was used. In all tests, wells of 3 cm in diameter in tissue culture plates, were seeded with 2×10^5 cells/well in 3 ml of Hanks' minimal essential medium (MEM) supplemented with 1.0 mM L-glutamine, 40 U/ml penicillin, 40 μg/ml streptomycin, and 10% fetal calf serum (FCS) and incubated for 72 h at 37°C in a humidified atmosphere of 5% CO₂ in air, to obtain a confluent monolayer of cells. Y1 mouse adrenal cortex tumor cells (ATCC collection) were grown in 1.5-diameter wells in 1 ml Earle's MEM supplemented with 10% FCS to obtain confluent monolayers of $\sim 10^5$ cells/cell. All tissue cell monolayers were washed twice with phosphate-buffered saline (PBS; 0.02 M phosphate and 0.15 M NaCl; pH 7.4) before assay.

Tissue culture assay system. Stationary phase bacteria were diluted in fresh trypticase soy broth medium, grown to a late log phase, centrifuged, and washed twice with PBS. Fim⁺ bacteria were suspended in this buffer to obtain a cell density reading of 0.5 OD₅₄₀ ($\sim 5 \times 10^8$ bacteria/ml). 1 ml of the suspension was added to each well containing washed tissue cells and after 30 min, the supernatant was decanted and the monolayers were washed five times with PBS. 3 ml of Hanks' MEM (without antibiotics) were then added to each well.

In separate experiments to test the effects of nonadherence, Fim⁻ bacteria were suspended in Hanks' MEM and diluted in the same medium to $\sim 10^7$ bacteria/ml; 3 ml of the diluted suspension were added to

Address reprint requests to Dr. Eisenstein, whose present address is Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109.

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1. Abbreviations used in this paper: cAMP, cyclic AMP; CFU, colony-forming units; Fim⁺, fimbriated; Fim⁻, nonfimbriated; IBMX, isobutylmethylxanthine; ¹²⁵I-TSME, ¹²⁵I-iodotyrosylmethylsuccinyl ester; LT, heat-labile enterotoxin; MEM, minimal essential medium.

washed tissue culture cell monolayers. Test agents were included in the Hanks' MEM diluent. All bacteria-tissue culture cell mixtures were incubated at 37°C at constant shaking for a total period of 5-6 h to determine cAMP concentrations and amount of bacterial growth.

Determination of bacterial growth. In these experiments controls wells, lacking tissue culture cells but otherwise treated the same, were included. In addition, in one set of experiments, 10 µg/ml proline was added to Hanks' MEM diluent. 100-µl samples of supernatant were taken from wells containing Fim⁻ bacteria with tissue culture cells and from wells lacking tissue culture cells at 1-h intervals for viable counts by plating on nutrient agar and determining colony-forming units (CFU). In the case of Fim⁺ bacteria, which, unlike the Fim⁻ bacteria, were adherent to the tissue culture cells, the medium was decanted at 1-h intervals and the cell monolayer was washed twice with PBS and then treated with 4% saponin with vigorous shaking to remove the tissue cells from the plastic and dissociate the adherent bacteria. The viable count was then determined in the saponin wash. Saponin had no effect on the viability of the organisms (data not shown).

In a separate set of experiments, bacterial growth was monitored spectrophotometrically at 540 nm. In these experiments, the growth medium was either fresh Hanks' MEM supplemented with various concentrations of proline, or Hanks' MEM not supplemented with proline and preincubated for 5 h on tissue culture cells, designated conditioned medium. Washed stationary phase bacteria were seeded into 2 ml of the growth media and incubated for 24 h at 37°C.

cAMP determination. cAMP was determined by radioimmunoassay, essentially as described by Steiner (6) and Harper and Brooker (7).

After aspiration of the medium, tissue culture cells in each well were resuspended in cold 5% TCA for 30 min, sonified in a Branson 3-A sonifier for 2 min, and the precipitate removed by centrifugation. TCA was removed from the extract by four extractions with 2 ml water-saturated diethyl ether and the ether was removed by 5 min heating at 60°C and subsequent brief application of low vacuum. The extract was diluted to a desired concentration and was acetylated with acetic anhydride in the presence of triethylamine. A 50-µl aliquot of the acetylated extract was incubated, with anti-cAMP antibody (dilution 1:60,000) and ~ 10⁴ cpm of ¹²⁵I-iodotyrosylmethylsuccinyl ester of cAMP (¹²⁵I-TSME-cAMP) for 14-18 h at 4°C. After incubation, the bound ligand was separated from the free by adsorption on activated charcoal. Values were calculated from a standard curve by a curve-fitting program supplied by a Compu-gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Determination of cAMP in the bacteria-tissue cells supernatant was performed essentially in the same way, except that the sonication step was omitted. To ensure the validity of the results, serial dilutions were assayed, as well as internal standards.

Determination of LT activity. 0.5-ml aliquots of various bacterial suspensions or culture supernatants were added to a confluent Y1 mouse adrenal cell monolayer with and without anti-LT toxin and incubated for 30 min at 37°C. The monolayers were then washed three times with PBS and incubated in 1 ml Earle's MEM without antibiotics for 4 h at 37°C. LT activity was determined as the percentage of rounded cells in several representative fields (8). The amount of adherent bacteria was determined on stained monolayer microscopically by counting the number of adherent bacteria on 100 cells and multiplying by 10³ to obtain the number of adherent bacteria per well.

Preparation of anti-cholera toxin. Pure cholera toxin (Swiss Serum and Vaccine Institute, Berne, Switzerland) was suspended in saline to a concentration of 400 µg/ml and mixed 1:1 with complete Freund adjuvant. Rabbits were injected intramuscularly with the mixture and bled as described previously (9). These antisera were used as anti-LT toxin given the known strong antigenic crossreactivity between the two toxins (10).

Chemicals and media. Anti-cAMP antibody was a generous gift of Dr. G. Brooker of Georgetown University. ¹²⁵I-TSME-cAMP was synthesized and purified in our laboratory. Tyrosylmethylsuccinyl cAMP was the product of Sigma Chemical Co. All other chemicals and media used in these studies were of the highest quality and purity available.

Results

In preliminary studies to determine the effect of bacterial fimbriation on tissue cell adherence, wells containing tissue cell monolayers were exposed to suspensions of the Fim⁺ and Fim⁻ strains of *E. coli* in PBS for 30 min at 4°C followed by washing to remove nonadherent bacteria. We found that about 1 × 10⁷ CFU of *E. coli* cells adhered to the tissue cell monolayer exposed to 5 × 10⁸ CFU of Fim⁺ *E. coli* and that this adherence was significantly (> 90%) blocked by α-methyl-D-mannoside, confirming that the Fim⁺ bacteria adhere via mannose specific adhesins (Type 1 fimbriae) to the intestinal cell monolayer (11). In contrast, < 10⁶ CFU of Fim⁻ cells adhered to the tissue cell monolayer. In subsequent experiments with tissue cell monolayers, therefore, the adherent Fim⁺ bacteria after wash contained ~ 10⁷ CFU per monolayer. Consequently, the subsequent biochemical assays of the Fim⁺ bacteria were performed by adding to the bacteria-adherent monolayers sterile test medium, whereas assays of the Fim⁻ bacteria were performed by first diluting the bacterial suspension in the test medium and then adding it to the tissue cell monolayers in equal final volumes that contained 10⁷ CFU.

Growth of bacteria on intestinal cell monolayers. The growth of Fim⁻ and Fim⁺ stains in MEM medium was proline dependent in wells devoid of cell monolayers (Table I). In contrast, when both bacterial strains were allowed to grow in wells containing intestinal cell monolayer in proline-free medium, there was ~ 10-fold increase in CFU during the 6-h growth period (Fig. 1). There was, however, a difference in the lag period between the two strains. While the Fim⁺ organisms started to grow immediately, the nonadherent Fim⁻ organisms started to grow 2 h after their inoculation to the wells. The Fim⁺ bacteria actually started to grow sooner in wells with tissue cells and proline-deficient medium than they did in proline-supplemented medium without the tissue cells (Table I). Such differences were not seen for the Fim⁻ organisms. The longer lag observed in Fim⁻ bacteria grown on cell monolayers alone, as compared with that in the presence of monolayers and proline, represents the period required for the proline substitute to reach critical concentrations to enable the initiation of growth in the bulk medium. It should be noted that there was a difference, under the experimental conditions, in the duration of the lag period

Table I. Dependence of the Growth of Fim⁺ and Fim⁻ Proline Auxotrophs of *E. coli* on Tissue Culture Cells and Proline

Content of wells in addition to MEM Hanks' medium		Fim ⁺	Fim ⁻		
Tissue cells	Proline (10 µg/ml)	Generation time*	Lag phase	Generation time	Lag phase
		min	min	min	min
Absent	not added	no growth		no growth	
Absent	added	42.6±7.0	56.5±8.0	43.7±0.7	125.0±12
Present	not added	37.9±1.3	2.0	42.3±4.0	100.5±11
Present	added	42.3±1.8	2.0	45.2±8.0	41.7±2.6

*The generation (doubling) time was calculated from the slope of the steepest section of the growth curves obtained during 6 h that gave a correlation coefficient of >0.98 (see Fig. 1).

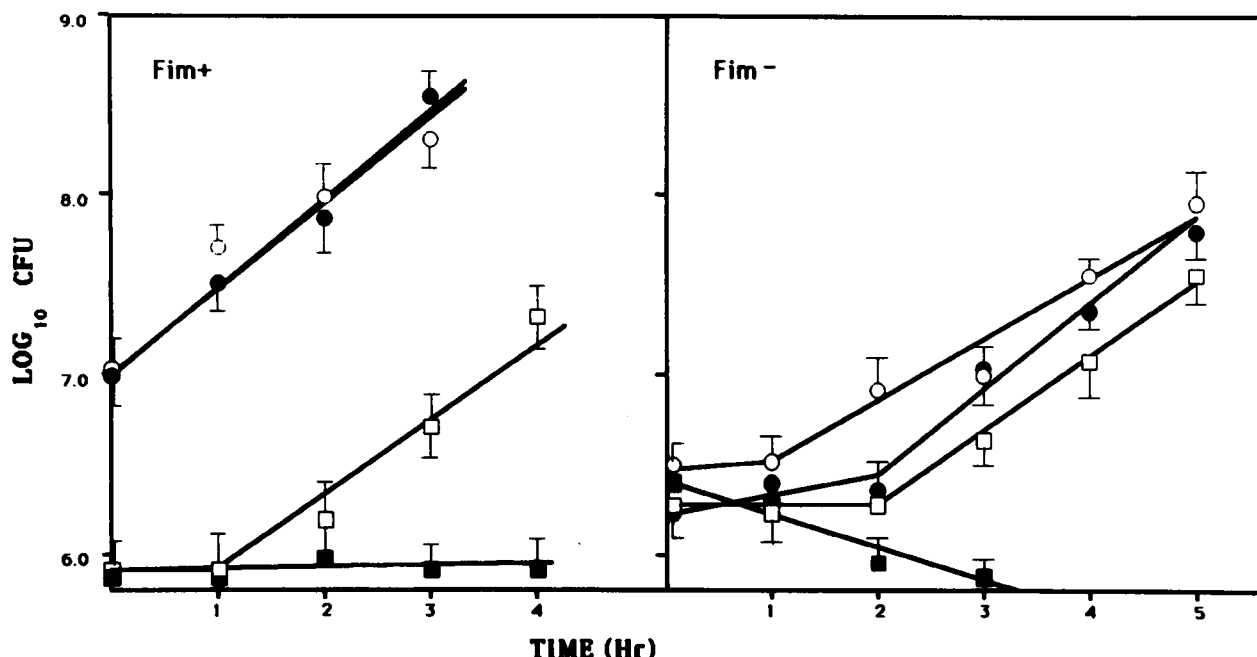


Figure 1. Effects of CCL6 tissue culture cells on growth of Fim⁺ (CHS50) and Fim⁻ (VL584) strains of *E. coli*. Determination of bacterial growth was done in wells containing 3 ml of Hanks' MEM without (squares) or with (circles) tissue culture cell monolayer that were either supplemented (open symbols) or unsupplemented (solid symbols) with 10 μ g/ml proline.

between the Fim⁻ and Fim⁺ variants in wells devoid of tissue cells but containing medium supplemented with proline (Table I). We did not see this difference, however, when the organisms were grown in wells containing proline and α -methyl-D-mannoside (20 μ g/ml), or in glass tubes containing proline. Thus, in the absence of tissue cells the different length of lag phase (i.e., 56.5 vs. 125 min) probably reflects the effect of mannose-sensitive interaction between the plastic wells and the fimbriate bacteria.

In another set of experiments, the Fim⁺ and Fim⁻ strains were grown in wells containing tissue cells and enough α -methyl-D-mannoside (20 μ g/ml) to prevent attachment of Fim⁺ bacteria to the tissue cells. The duration of the lag phase for the Fim⁺ and Fim⁻ strains under these conditions was identical, 1 h in proline- (10 μ g/ml) supplemented medium and 2 h in unsupplemented medium, suggesting that the two isogenic strains differ only in fimbriation. Thus, any growth advantage of the Fim⁺ variant is due solely to its enhanced ability to attach to the tissue cells.

To see whether the growth factor secreted by the tissue cells is mainly proline, we used two additional proline auxotrophic strains of *E. coli* (AB 2659 and Met F3455) and compared the growth of the bacteria in Hanks' MEM with that in tissue-conditioned medium. The growth of the proline auxotrophic strains in conditioned medium not supplemented with proline was equivalent to that of medium supplemented with 2 μ g/ml proline (Table II). The amino acid analysis of fresh medium showed no detectable proline, whereas that of conditioned medium (i.e., supernatant incubated 5½ h in wells containing tissue culture cells) showed 1.6 μ g/ml proline. The results suggest that proline is indeed one of the growth factors secreted by the tissue cells that is required by all three proline auxotroph strains.

When the Fim⁻ organisms were cultured in the presence of both proline and tissue cells, the lag period was shorter than when grown in the presence of proline alone. To determine

whether the tissue cells secrete growth factors other than proline, we performed similar experiments to those described in Table I with Pro⁺ derivatives of the Fim⁺ and Fim⁻ isogenic variants (VL798 and VL799, respectively), instead of the parent Pro⁻ strains. We found that the Fim⁻ started to grow 1 h after inoculation while the Fim⁺ strain started to grow immediately. The results suggest that tissue cells provide factors other than proline that promote growth.

Table II. Growth of Proline Auxotrophic Strains of *E. coli* in either Proline-supplemented or Conditioned Medium*

<i>E. coli</i> strain	Conditioned medium	Proline	OD ₅₄₀ after 24 h
		μ g/ml	
CSH50	-	0	0
	-	1	0.02
	-	2	0.035
	-	4	0.07
	-	5	0.09
	-	10	0.15
AB2659	+	0	0.03
MetF3455	+	0	0.029
CSH50	+	0	0.03

*3 ml of conditioned medium derived from 5 h of incubation with tissue culture cells (CCL6) of Hanks' MEM, not supplemented with proline, were inoculated with the various Pro⁻ strains. After 24 h at 37°C the total bacterial mass was determined spectrophotometrically. A representative growth under the same conditions of strain CSH50 in Hanks' MEM supplemented with various concentrations of proline is included.

Table III. Distribution of cAMP Secreted by the Various *E. coli* K-12 Strains Grown with Intestinal Tissue Culture Cell Monolayer

<i>E. coli</i> strain	Medium	Tissue cell monolayer	cAMP associated with tissue cell monolayers
	pmol cAMP/well*	pmol cAMP/well*	% total
CSH50 (Fim ⁺)	190.0±29.5	69.5±9.3	26.8±2.64
VL584 (Fim ⁻)	104.5±31.0	0.08±0.2	0.8±0.19
VL391 (Fim ⁺ Cya ⁻)	<1.0	<1.0	
Culture filtrate of CSH50 (Fim ⁺)	1070±50.0	20.0±1.34	1.8±0.1

*cAMP, associated with tissue culture cells and in IBMX-containing medium was determined after 6-h incubation with the indicated *E. coli* strains. The low, control values of cAMP (2–5 pmol cAMP/well) associated with tissue culture cells not exposed to bacteria but treated under identical conditions were subtracted from experimental values obtained in the same experiment.

Diffusion of bacterial cAMP from the monolayer-medium interface. The above results suggest that Fim⁺ *E. coli* bacteria have a growth advantage over the Fim⁻ bacteria in wells containing intestinal tissue cell monolayer, possibly because of the increased proximity of the adherent bacteria to a high concentration of the nutrient source. To test the hypothesis that such proximity places these adherent bacteria in a region where the concentrations of the product(s) of bacterial and tissue culture cells is elevated, we next examined the diffusion characteristics of an easily measured small molecule diffusing from the monolayer-medium interface. We chose bacterial cAMP, because it is known that *E. coli* bacteria secrete most of the cAMP they produce during their growth (12).

Both Fim⁺ and Fim⁻ derivatives produced identical amounts of cAMP, which was predominantly secreted into the medium (Table III). As expected, the Fim⁺ Cya⁻ mutant produced negligible amounts of cAMP. When the organisms were allowed to grow under identical conditions but in wells containing tissue cell monolayers, ~ 20–30% of the cAMP produced by the adherent (Fim⁺) bacteria remained associated with the tissue culture

cells as compared with only 0.8% of that produced by the isogenic, nonadherent (Fim⁻) bacteria. No detectable cAMP was measured in wells exposed to the Fim⁻ strain and washed free of nonadherent bacteria, which suggests that the negligible amount of nonspecific adherence of Fim⁻ bacteria did not produce detectable amounts of cAMP. To show that all of the cAMP was of bacterial origin, we repeated the experiments in the presence or absence of isobutyl-methylxanthine (IBMX), a specific inhibitor of cAMP phosphodiesterase activity in eukaryotic cells. We found no difference in cAMP levels between the treated and untreated cultures (data not shown). To demonstrate the efficiency of IBMX in this system, we added 40 µg/ml of cholera toxin to the medium (as an extrinsic stimulator of eukaryotic adenylate cyclase) in the absence or presence of IBMX. We found that the addition of IBMX under these conditions increased tissue culture cell cAMP > 16-fold (from 5 to 80 pmol/well). These results indicated that virtually all of the cAMP in the experiments described in Table III was of bacterial origin.

The above findings show that a significant fraction (up to 30%) of the total cAMP secreted by the adherent (Fim⁺) bacteria remained associated with the tissue cell monolayer in wells where the culture medium was aspirated without further washings of the cell monolayer. In contrast, when monolayers were instead exposed to bacteria-free culture filtrates, which contained as much as 1070 pmol cAMP, we found that only 1.8% of this amount remained associated with the cell monolayer (Table III). When cell monolayers were washed vigorously with glucose-containing medium (to repress new bacterial cAMP synthesis [13]), only 0.8% of the bacterial cAMP remained associated with the monolayer. In contrast, similar washing with glucose-free medium, which allows continued bacterial synthesis of cAMP (13), resulted in 25% of the total secreted cAMP associated with the monolayer. Thus the high concentration gradient of bacterial products at the monolayer interface required both adherent bacteria and continued bacterial synthesis.

LT toxin activity of E. coli adherent to Y1 mouse adrenal cells. The results described above suggest that the concentration of potentially toxic products, secreted by adherent bacteria, should be much higher in the vicinity of the tissue cells, as compared with that in the bulk of the medium. To test this hypothesis, we examined the biological activity of LT toxin secreted by bac-

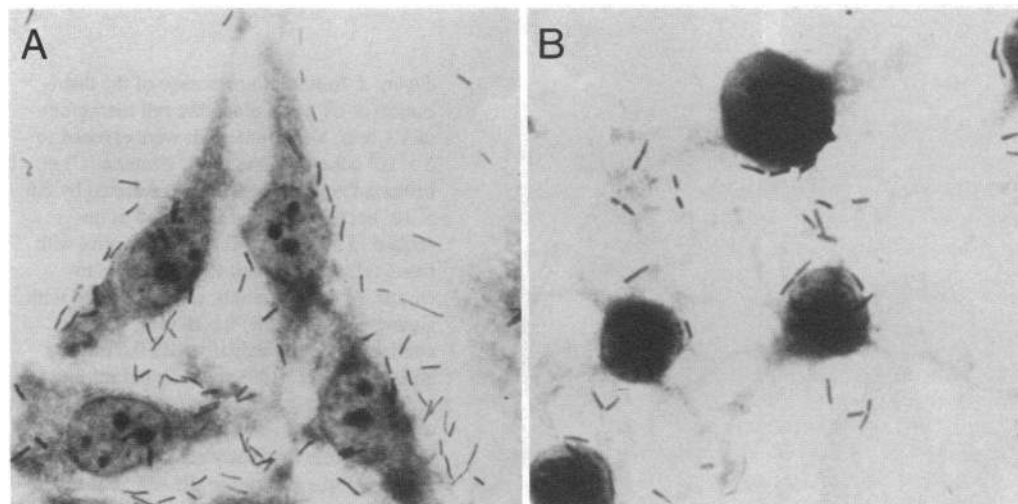


Figure 2. Rounding up of Y1 mouse adrenal cells by adherent Fim⁺ Tox⁺ derivative of *E. coli* strain CSH50. B, round Y1 cells containing adherent Fim⁺ Tox⁺ bacteria (Table III). A, control of Fim⁺ Tox⁻ *E. coli* strain CSH50 adherent to Y1 cells (Table III).

teria adherent to Y1 mouse adrenal cells, which round up upon exposure to the LT toxin (8). The LT secreted by the $Fim^+ Tox^+$ bacterial suspension caused rounding up of Y1 cells in a dose-dependent fashion (Table IV) with no detectable LT toxic activity in the supernatant of a suspension containing 6×10^6 bacteria. When $Fim^+ Tox^+$ bacteria were allowed to adhere before assay, however, as few as 2.6×10^5 bacteria adherent per monolayer caused $\sim 20\%$ rounding up of cells (Table IV). No detectable LT toxin activity was observed in conditioned medium derived from tissue cells containing as many as 1.6×10^6 adherent bacteria per well. Although there is some loss of LT toxin activity during transfer and incubation of conditioned medium (Table IV), this loss of activity cannot account for the lack of any detectable rounding up of cells in any of the conditioned media derived from tissue cells monolayers containing adherent $Fim^+ Tox^+$ bacteria. There was no detectable toxic activity in wells containing $Fim^+ Tox^-$ bacteria (Table IV; Fig. 2), indicating that the rounding up of Y1 cells containing adherent $Fim^+ Tox^+$ bacteria is due to LT toxin. Tissue cells were exposed to various amounts of $Fim^- Tox^+$ bacteria under similar experimental conditions. The number of nonadherent bacteria per well needed to be present to cause 20% rounding up of cells was as high as 1×10^7 (data not shown). When the nonadherent bacteria of the $Fim^- Tox^+$ strain were removed by washing and wells were further incubated, no LT toxic activity was detected, indicating that the negligible amount of nonspecific adherence was insufficient to cause any rounding up of tissue cells.

The distribution of LT toxin bound by tissue cells along the cell membrane was examined by indirect immunofluorescence. We noticed a homogeneous distribution along the cell membrane of Y1 cells exposed to soluble LT toxin, in contrast to a spotty distribution on cells exposed to adherent $Fim^+ Tox^+$ bacteria (Fig. 3), suggesting that only part of the cell membrane was exposed to the toxin.

Discussion

The aim of the present study was to examine two important physiological events subsequent to bacterial adherence to animal cells. We measured the availability of bacterial nutrients secreted

by tissue cells and the distribution of bacteria secretion products during growth of a fimbriate, proline-auxotrophic strain of *E. coli*. The results suggest that tissue cell cultures provide a proline substitute(s) that supports bacterial growth. The adherent (Fim^+) bacteria benefit more from such growth factors than nonadherent (Fim^-) bacteria, as shown by the absence of a lag phase before initiation of growth of the former. Because the growth of proline auxotrophic *E. coli* is dependent on a critical concentration of proline, it would appear that the concentrations of amino acids (and possibly other growth factors) in the space proximal to the monolayer is much higher than in the bulk of the medium. This concentration gradient could account for the early initiation of growth of the adherent bacteria and is reminiscent of food scavenging yielding a selective growth advantage of adherent marine bacteria (14).

Likewise, when concentration gradients of bacterial products were examined, about a third of the total amount of cAMP produced by the growing adherent bacteria remained associated with the tissue cell monolayer, while virtually all of the nucleotide secreted by nonadherent bacteria diffused into the medium. The fraction of cAMP that remained associated with the intestinal cell monolayer was produced by the adherent bacteria and not by the tissue cells because: (a) no cAMP could be detected in the medium by growing a $Fim^+ Cya^-$ derivative adherent to tissue cells, suggesting that the adherence of bacteria to the tissue cells alone does not trigger the latter to produce cAMP; (b) the cell-associated cAMP is readily washed by vigorous washing only with solutions that induce catabolite repression of cAMP synthesis by the bacteria; and (c) IBMX, a cAMP phosphodiesterase inhibitor, which we have shown to be capable of affecting cAMP levels of tissue-cell, but not of bacterial origin, had no effect on cAMP levels during the test. Thus, the increase in cell-associated cAMP was not the result of stimulated tissue cell production. The association of cAMP synthesized by Fim^+ bacteria with the cell monolayer is not the result of unique binding properties of this molecule, because only 1.8% of exogenously added cAMP remained associated with the cell monolayer and because adherent bacteria were unable to maintain the concentration gradient when cAMP production was slowed by adding glucose.

Unlike the small molecule cAMP, which is readily secreted

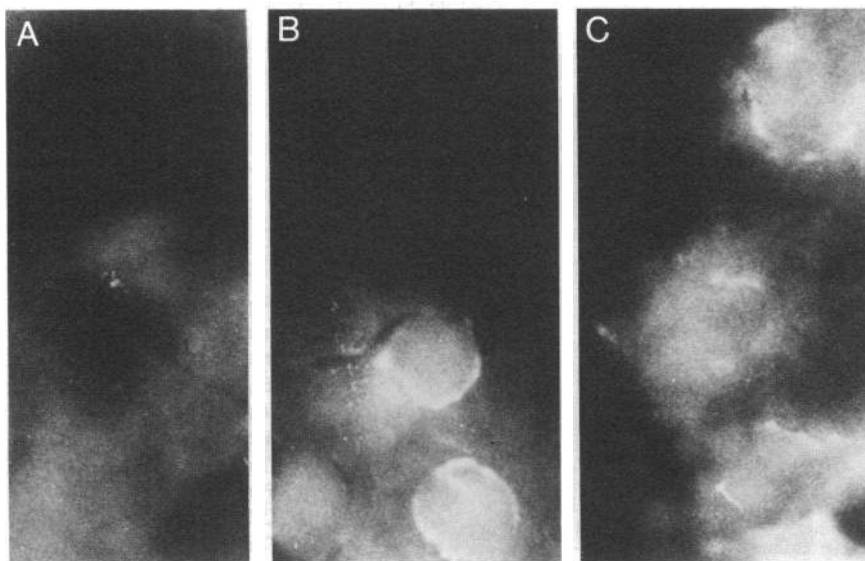


Figure 3. Immunofluorescence of the distribution of LT toxin along the cell membrane of Y1 cells. Y1 adrenal cells were exposed to 3×10^5 adherent $Fim^+ Tox^+$ bacteria (C) or bacteria-free, soluble LT toxin secreted by 2.6×10^7 bacteria (B), and incubated as described in legend to Table III. A control with tissue cells not exposed to toxin (A) is included. After incubation, cells were fixed with glutaraldehyde (0.5%) for 10 min, washed, and incubated sequentially with 0.5 ml of anti-LT toxin (1:100) and with 0.5 ml fluorescein-labeled goat anti-rabbit immunoglobulin (1:20) (Kallestad Laboratories, Inc., Austin, TX). Note the spotty staining of toxin on the tissue cells exposed to the adherent, toxin-producing bacteria (C).

by *E. coli* cells, and in contrast to the efficient secretion of cholera toxin in *Vibrio cholerae*, the analogous enterotoxin from *E. coli*, LT, is primarily cell associated (15). Nevertheless, small amounts of LT can be detected in supernatant fractions of growing *E. coli* cells (15). (Most of this extracellular LT is actually associated with membrane vesicles, which are steadily released from sections of newly synthesized outer membrane during *E. coli* growth [16]. Thus, although we are using the term secreted LT to denote conceptually the LT that is released by the cells, the process of LT secretion is complex.) Because *E. coli* is so inefficient at secreting LT, the small amount of enterotoxin liberated from the cells might need to be released, whether as free toxin or vesicle-packaged toxin, at an optimal site (i.e., adjacent to the target receptors) to cause toxic effects. The results described in Table IV indicate that the small amount of LT secreted by *E. coli* adherent to Y1 mouse adrenal cells is about 100 times more concentrated in the space proximal to the cells than in the bulk of the medium. This is most probably the reason for the toxic activity detected in wells containing the Y1 cells and adherent Fim⁺ Tox⁺ *E. coli*, but not in the conditioned medium derived from these wells. These results are in agreement with

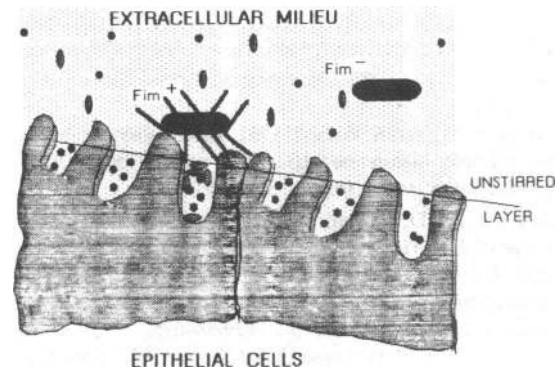


Figure 4. Schematic distribution of products secreted by epithelial cells (filled circle) and by bacteria (shaded oval) adherent to the cells, between the proximal and the distal milieus of the epithelial cell surfaces. The secretory products accumulate in pockets of the unstirred layer formed by the ruffle structure of the epithelial cell membrane and the lid of the adherent bacterium.

Table IV. Effect of LT Toxin Secreted by *E. coli* Adherent to Y1 Mouse Adrenal Cells*

Origin of LT toxin	Number of bacteria	Percent rounding cells exposed to		
Supernatant of Fim ⁺ Tox ⁺ suspension	8.1 × 10 ⁸	>90	>90	ND [†]
	1.0 × 10 ⁸	>90	70 ± 10	20 ± 5
	5.0 × 10 ⁷	70 ± 10	50 ± 10	10 ± 5
	2.6 × 10 ⁷	50 ± 10	20 ± 5	5 ± 2
	6.0 × 10 ⁶	<5	ND	ND
Adherent bacteria	Fim ⁺ Tox ⁺	Conditioned medium	Fim ⁺ Tox ⁻	
	1.6 × 10 ⁶	>90	<5	<5
	5.0 × 10 ⁵	75 ± 10	<5	<5
	3.0 × 10 ⁵	55 ± 10	<5	<5
	2.6 × 10 ⁵	20 ± 5	<5	<5

To obtain monolayers with various amounts of indicated adherent bacteria before assay of LT toxin activity, the Y1 cell monolayers were preexposed to the bacterial suspensions containing various amounts of bacteria and constant amount of anti-LT toxin (1:200) to neutralize any presecreted toxin. After incubation at 37°C for 30 min, the monolayers were washed free of nonadherent bacteria and anti-LT toxin, followed by adding fresh medium. After 4 h incubation, the percent rounding up of cells was determined and the medium designated conditioned medium was transferred to fresh monolayers to determine LT toxin activity after 4 h incubation at 37°C. The number of adherent bacteria per well was determined microscopically on stained monolayers.

*Wells containing tissue monolayers of Y1 cells were exposed to the supernatants derived from bacterial suspensions containing various amounts of indicated bacteria. The percent of rounded cells was determined after 4 h incubation at 37°C and the medium designated and conditioned medium was transferred to fresh monolayer to determine LT toxin activity after 4 h incubation at 37°C.

the distribution of cAMP secreted by adherent bacteria. Prior workers have suggested that the role of bacterial adhesive factors in promoting enterotoxigenic *E. coli* diarrhea is primarily to permit bacterial multiplication and persistence in the bowel (17). Our findings here directly support the conjecture of Witholt and co-workers (3, 16) that the additional effect provided by bacterial adherence factors of enhanced toxin delivery may also be important in the production of clinical disease.

We interpret our results by postulating that certain products secreted by either the tissue cells or the cell-adherent *E. coli* accumulate in crypts formed by the ruffle structures of the animal cells and the lids formed by the adherent bacteria. This trapped or unstirred layer limits the diffusion of such products (including vesicle-packaged toxin) to the surrounding medium, as Fig. 4 depicts. This hypothesis may explain both the nonhomogeneous distribution of cAMP secreted by growing and adherent *E. coli* bacteria, the enhanced toxic activity caused by LT-secreting adherent bacteria, the spotty distribution of LT toxin along the cell membrane of Y1 cells exposed to Fim⁺ Tox⁺ bacteria, and the growth advantage of the adherent bacteria resulting from the proximity of the organisms to relatively high concentrations of nutrients accumulated in such crypts (Fig. 4). This model is also compatible with the concept of an unstirred layer found to occur in intestinal tissue cells (18). It is, therefore, conceivable that growth-limiting factors secreted by the host cells and toxic products secreted by the bacteria in vivo in an open system would reach effective concentrations in the case of adherent but not in the case of nonadherent bacteria, thereby increasing the pathogenicity of the adhering phenotype.

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