INFECTION AND IMMUNITY, Aug. 1997, p. 3474–3477
0019-9567/97/$04.00+0
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Vol. 65, No. 8

# Adherence of *Vibrio cholerae* to Cultured Differentiated Human Intestinal Cells: an In Vitro Colonization Model

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Received 10 March 1997/Returned for modification 23 April 1997/Accepted 20 May 1997

**Choleragenic vibrios adhered to and multiplied on monolayers of the highly differentiated mucin-secreting cell line HT29-18N2. Their adherence followed first-order kinetics, was dependent on the concentration of vibrios, and was partially inhibited by lipopolysaccharide. Comparison of genetically modified vibrios showed that flagella, an active *toxR* gene, and the virulence cassette were not essential for initial binding. Inactivation of the hemagglutinin/protease increased binding. This highly differentiated human intestinal cell line provides a versatile new approach for studying major events occurring during intestinal colonization: adherence, multiplication, and detachment.**

To cause cholera (14), *Vibrio cholerae* organisms must pass the gastric acid barrier, colonize the human small bowel, and liberate enterotoxin. Subsequently, the vibrios detach (6, 19). Intestinal colonization results from the capacity of the infecting vibrios to persist and multiply in the small bowel. Genes that regulate intestinal colonization can be putatively identified by mutations that cause a defect in bacterial colonization in infant mice, other animal models, or cultured cells. However, it is not clear that any of these surrogate models is completely suitable to evaluate the factors that are essential for colonization of the human gut.

An early approach to identify putative adhesins exploited the capacity of vibrios to agglutinate erythrocytes from different sources (4). Putative adhesins included soluble hemagglutinin/protease (Hap) and the following cell-associated hemagglutinins: mannose-sensitive hemagglutinin (MSHA), fucose-sensitive hemagglutinin, and a mannose-fucose-resistant hemagglutinin (4). The capacity of vibrios to bind cultured human intestinal (Intestine 407) cells in vitro correlated with expression of several hemagglutinin activities (3), while Hap was suggested to mediate detachment (6, 9). However, expression of the above-mentioned hemagglutinins in vitro does not always correlate with intestinal colonization. For example, although MSHA was previously regarded as an important biotype-specific colonization factor (20), inactivation of the *mshA* gene, encoding the type IV mannose-binding pilus subunit (13), did not significantly diminish intestinal colonization in suckling mice (1, 26).

It should be recognized that the cells used previously to identify putative adhesins differ structurally and functionally from the highly differentiated mucus-coated cells lining the human intestinal mucosa. Enterocytes and goblet cells are the major cell types lining the human intestinal mucosa (5, 22). A clone, HT29-18N2, derived from the human colonic adenocarcinoma HT29 cell line, has been shown to differentiate in a glucose-containing protein-free medium into columnar monolayers of predominantly goblet cells filled with mucous secretory granules (11, 21).

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In this work, we examine the interactions between *V. cholerae* O1 (of both biotypes and serotypes) and *V. cholerae* O139 with differentiated HT29-18N2 cells. We show that these interactions mimic important events accompanying intestinal colonization: adherence, growth of infecting vibrios, and detachment. Enterotoxigenic *Escherichia coli* strains have been previously shown to adhere to brush borders of a different mucin-secreting subpopulation of HT29 cells (16).

*V. cholerae* strains were grown as semiconfluent streaks on meat extract agar (MEA) consisting of Bacto Beef Extract (3 g/liter), Bacto Peptone (10 g/liter), NaCl (5 g/liter), and Bacto Agar (20 g/liter) (pH 7.6) at 37°C. The HT29-18N2 cell line was provided by D. Louvard (Pasteur Institute, Paris, France) and grown as described previously (21). Cells were seeded onto 13-mm-diameter cover glasses in 24-well tissue culture plates in high-glucose Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 5% dialyzed fetal bovine serum. Twenty-four hours after seeding, the cells were induced to differentiate by switching to protein-free hybridoma medium (PFHM-II; Gibco) for 10 to 14 days.

Vibrios were harvested in PFHM-II at 16 h, diluted, and placed on confluent monolayers of approximately $10^6$ differentiated HT29-18N2 cells. After incubation at 37°C for appropriate times, triplicate cover glasses were washed three times with phosphate-buffered saline, pH 7.4, to remove unbound bacteria and transferred to a new well containing phosphate-buffered saline plus 1% Triton X-100 to lyse the HT29-18N2 cells. The cover glasses were rocked in a Micro Shaker II (Dynatech Products) for 30 min at room temperature, and the suspension was thoroughly mixed by repeated pipetting. Vibrios retained full viability throughout these manipulations. The titer of immobilized vibrios was determined by serial dilution and plating of 10-μl droplets in triplicate on MEA. Since the degree of the HT29-18N2 differentiation process is difficult to control precisely between different seedings, important comparisons were made simultaneously on the same batches of HT29-18N2 monolayers.

Two types of experiments were performed. In one kind, referred to as dip experiments, cover glasses were exposed to $10^6$ to $10^7$ vibrios, usually for 30 min, washed, transferred to fresh (sterile) PFHM-II, and incubated for different lengths of time, and then the adherent vibrios were counted. Results

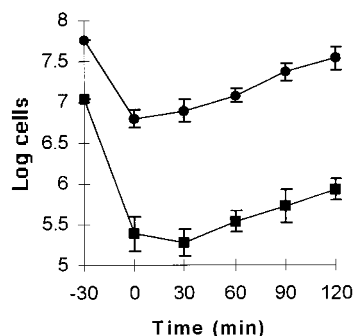


FIG. 1. Levels of adherence and growth of classical strain O395 (■) and El Tor Ogawa strain C7258 from Peru (●) on HT29-18N2 cells. In a dip experiment, cover glasses were preincubated with vibrios for 30 min, washed, and incubated in sterile PFHM-II. At each time point shown, three cover glasses were withdrawn and the mean titer of adherent vibrios was determined by dilution counting. The −30- and 0-min points in this graph correspond to the inoculum size and the number of vibrios which adhered, respectively, while subsequent time points show growth of the vibrios adherent to the monolayers. Error bars, standard errors of the means.

from an experiment of this type are shown in Fig. 1. Strains O395 (classical Ogawa) and C7258 (El Tor Ogawa) (Fig. 1) bound to the cover glasses during the initial 30-min exposure and subsequently grew, with doubling times of 41 min and 42.3 min, respectively. In the saturation experiments, monolayers of HT29-18N2 cells were exposed for 30 min to increasing amounts of vibrios. As shown for strain O395 (Fig. 2A) and C7258 (Fig. 2B), we did not observe a tendency toward saturation. Rather, adherence of the vibrios showed first-order-kinetic behavior which was dependent on their concentration.

In preliminary experiments, pretreatment of HT29-18N2 cells with 100 μg of lipopolysaccharide (LPS) purified from strain 3083 (El Tor Ogawa) (7) inhibited the adherence of $4 \times 10^6$ 3083 cells by 64%. The inhibition was reduced to 30 and 6%, respectively, by increasing the inoculum by 1 or 2 log units. LPS from *E. coli* was less effective (35%) with $10^6$ vibrios. These results suggest that LPS might play a role in adherence of *V. cholerae*. LPS is known to bind mucus (12). It is noteworthy that, at least until the discovery of O139 in 1992, only two LPS serotypes out of 150 *V. cholerae* serogroups were responsible for all the epidemic cholera in the world.

The proportion of the inoculum that adhered remained constant over a wide range of vibrio concentrations (Fig. 2). In preliminary experiments, we examined the possibility that this fraction could reflect a subpopulation of bacteria competent for adherence by assessing the capacity of vibrios recovered from monolayers to bind HT29-18N2 cells again. However, vibrios previously bound to monolayers showed reduced binding, rendering the hypothesis unlikely (data not shown).

HT29-18N2 cells incubated with *V. cholerae* C7258 were examined by light microscopy, transmission electron microscopy, and scanning electron microscopy (SEM). For light and transmission electron microscopy monolayers were fixed and embedded in EMbed812 as previously described (8). As noted earlier in rabbit models (19), *V. cholerae* bound these cells in hot spots (Fig. 3A). The significance of this observation is not clear. Hot spots could be mucus buds to which vibrios are chemotactically attracted (5, 22). The higher density of vibrios at hot spots could also be due to vibrio-vibrio recruitment, adherence, or autoagglutination, the final of these processes being a phenomenon associated with the expression of type IV pili (MSHA or toxin-coregulated pili [TCP]) in gram-negative bacteria. This vibrio-vibrio adherence possibly could prevent saturation of monolayers (Fig. 2).

For SEM, monolayers incubated with vibrios were fixed in 2% glutaraldehyde–1% osmium tetroxide, gradually dried in ethanol, critical point dried, coated in a sputter coater with a thin layer of gold and palladium, and imaged in a scanning electron microscope. SEM (Fig. 3B) revealed the presence of vibrios in close association with HT29-18N2 brush borders.

The adherence of different wild-type and genetically modified *V. cholerae* strains is summarized in Table 1. Strain 413, derived from C6706 by deleting its virulence cassette (2), behaved identically to its wild-type parent. We conclude that the virulence cassette is not required for adherence of *V. cholerae* to HT29-18N2 monolayers. Strain JJM43, a ToxR$^-$ derivative of classical O395 (10), showed slightly—but not statistically significantly ($P = 0.05$ [*t* test])—reduced binding. This observation suggests that proteins encoded by members of the *toxR* regulon, e.g., TCP (10) and OmpU (25), play only a partial role in this initial stage of the colonization process. Peru-15 is a nonmotile, nonflagellated, virulence cassette-less derivative of C6709 (15). Nonmotile strains were hypothesized to be unable to penetrate the mucus barrier and thus unable to induce a local inflammatory response by interaction with the underlying enterocytes (18). No difference was observed between Peru-15 and its wild-type parent, indicating that motility and flagella are not required for initial binding to monolayers. We are presently comparing the subsequent localization of fluorescently labeled motile and nonmotile vibrios on HT29-18N2 monolayers. It is noteworthy that a nonencapsulated mutant of an O139 strain adhered as well as its parent, even though anticapsular antibody has been shown to be protective (24). In comparison with other cholera vibrios tested, strain 569B did not bind significantly to HT29-18N2 monolayers. It is known to be a poor colonizer in animal models and volunteers (17).

We have also tested the binding capacity of *hap* mutants. In strain HAP-1, the wild-type *hap* gene of 3083 was replaced by a *hap*::Tn*5* allele (9). In Fig. 4A we show that HAP-1 bound HT29-18N2 monolayers more than its *hap*$^+$ parent. Similar results were obtained with a *hap* mutant isolated from strain 413 by insertion of a *celA* reporter gene (23) (data not shown). The HAP-1 strain also appeared to grow slightly faster on HT29-18N2 monolayers than its wild-type parent (Fig. 4B). Adherence studies using Intestine 407 cells earlier provided evidence that the *hap* gene encodes a detachase activity (6, 9). Recently, *hap* mutants were shown to adhere and colonize in infant mice better than their *hap*$^+$ parents (23). A working

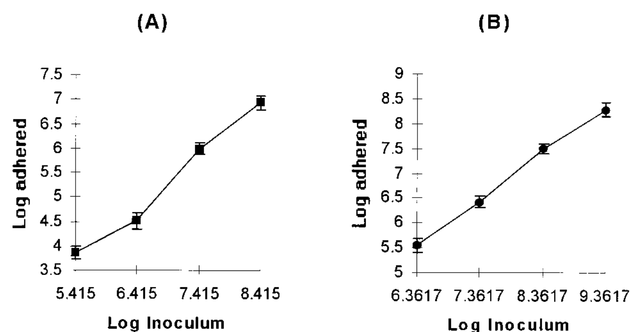


FIG. 2. Levels of adherence of different inoculum sizes of vibrios (O395 [A] and C7258 [B]) to HT29-18N2 cells. In a saturation experiment, cover glasses with HT29-18N2 cells were exposed to varying amounts of vibrios for 30 min, the cells were lysed, and the vibrios were counted. Error bars, standard errors of the means.

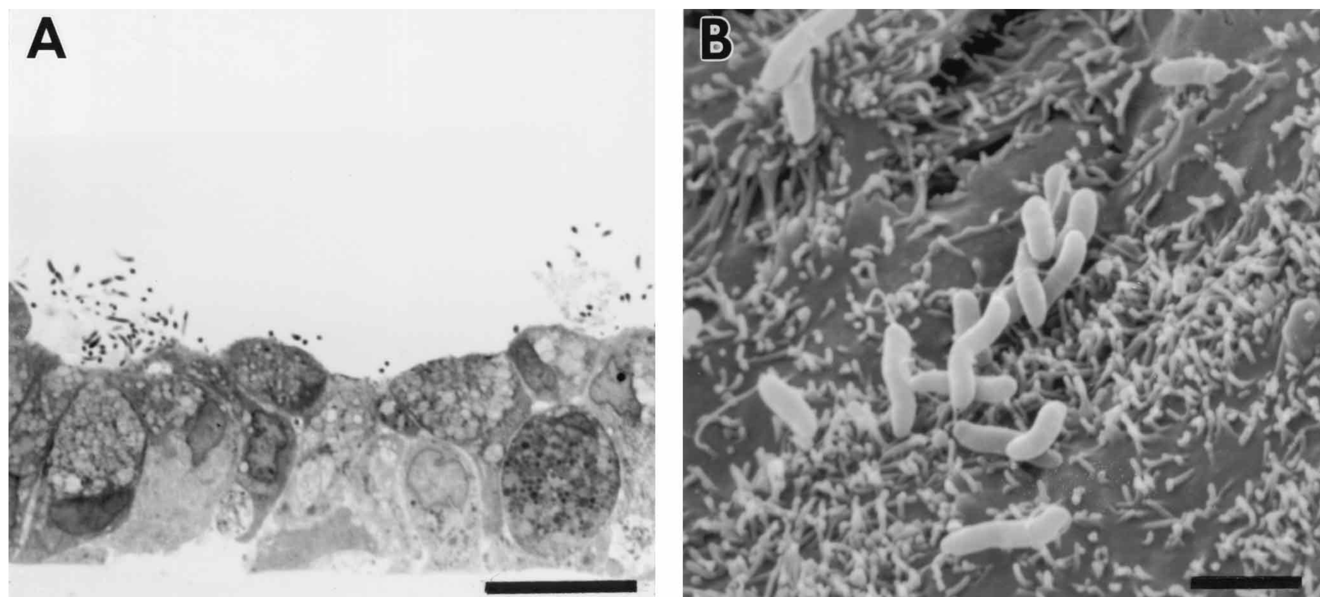


FIG. 3. Adherence of the El Tor biotype strain C7258 to monolayers of HT29-18N2 cells at 1 h. *V. cholerae* organisms adhere in clusters at hot spots scattered across the apical surface of the goblet cell monolayers as seen in light microscopy (A) or SEM (B) cross sections. (A) Bar = 20 μm; (B) bar = 2 μm.

hypothesis is that the mucinase activity of Hap causes sloughing of vibrios superficially attached to HT29-18N2 monolayers. Counting the vibrios released into the supernatant in Fig. 4B showed that at 120 min, the fractions of vibrios detached were 27 and 11% for 3083 and HAP-1, respectively, suggesting that Hap is not the only detachment factor in *V. cholerae*. Reintroducing the *hap* gene on plasmid pCH2 (9) reduced binding significantly (50%) (Fig. 4A) at 120 min, but the transformant still adhered better than the wild-type *hap*$^+$ strain.

A large number of putative colonization factors have previously been identified (14). Former studies, using erythrocytes and undifferentiated cell lines, emphasized the importance of individual adherence factors. The present study suggests that intestinal colonization is a multifactorial process involving a hierarchy of events in which vibrios in the intestinal lumen overcome the mucus barrier, adhere to mucosal surfaces, increase their population, and detach. Cultured HT29-18N2 cells are a close approximation to the mucus-coated differentiated cells lining the human intestinal mucosa. They provide a versatile, new, in vitro colonization model for experimental analysis of major events occurring during intestinal colonization: adherence, localization, multiplication, and detachment of infecting vibrios. The results obtained by SEM very closely resemble those reported earlier (27), which showed *V. cholerae* O139 adhering to formalin-fixed human intestinal biopsy samples.

TABLE 1. Percentages of adherence of different *V. cholerae* and mutant strains to HT29-18N2 cells

| Strain | Relevant characteristics and reference(s) | % Adherence[a] |
|---|---|---|
| C6706 | Wild type, El Tor, Inaba, from Peru (2) | 2.61 ± 1.14 |
| 413 | Δ(*cep orfU ace zot ctxA ctxB*) derivative of C6706 (2) | 2.63 ± 0.49 |
| O395 | Wild type, classical, Ogawa, from Bangladesh (10) | 2.25 ± 0.81 |
| JJM43 | *ctxA*-, *toxR*-negative derivative of O395 (10) | 1.07 ± 0.31 |
| C6709 | Wild type, El Tor, Inaba, from Peru (15) | 1.80 ± 0.08 |
| Peru-15 | Δ(*cep orfU ace zot ctxA ctxB*) nonmotile derivative of C6709 (15) | 1.88 ± 0.43 |
| MDO-12-C | Wild type O139, heavily encapsulated, from Bangladesh (7) | 1.04 ± 0.21 |
| MDO-12-C-41 | Nonencapsulated Tn*5* insertional mutant of MDO-12-C (7) | 1.20 ± 0.46 |
| 569B | Wild type, classical, Inaba, from India (17, 19) | 0.023 ± 0.005 |

[a] Cover glasses containing approximately $10^6$ HT29-18N2 cells were inoculated with $10^8$ to $10^9$ vibrios, incubated 15 min, and washed, and the titer of adherent vibrios was expressed as a percentage of the inoculum ± standard deviation ($n \geq 3$). Comparisons between strains C6706 and 413, O395 and JJM43, C6709 and Peru-15, MDO-12-C and MDO-12-C-41, and O395 and 569B were performed simultaneously (i.e., on the same lots of HT29-18N2 cells). Strains MDO-12-C and MDO-12-C-41 were incubated for 30 min prior to washing.

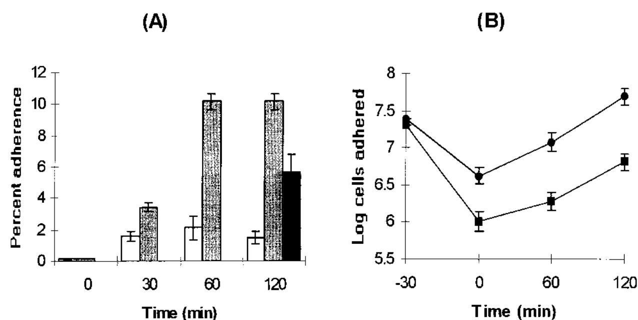


FIG. 4. Role of Hap in adherence and growth of *V. cholerae* on HT29-18N2 cells. (A) Approximately $10^6$ HT29-18N2 cells grown on cover glasses were incubated with $2 \times 10^9$ cells of strain 3083 (open bar), $1.33 \times 10^9$ cells of HAP-1 (shaded bar), or $1.08 \times 10^9$ cells of HAP-1/pCH2 (solid bar), and adherent vibrios were counted at different times. (B) In a dip experiment, cover glasses with HT29-18N2 cells were exposed for 30 min to $10^7$ cells of 3083 (■) or HAP-1 (●), washed, and incubated for 120 min in sterile PFHM-II. At each time point shown, three cover glasses were withdrawn and the titers of vibrios on the cover glasses and in the supernatants were determined by dilution plating.

This work was supported in part by U.S. Public Health Service grant AI 17312 to R.A.F. and a University of Missouri Research Board grant.

We are grateful to Victor DiRita and John Mekalanos for providing strains JJM43 and Peru-15, David Morrison for providing *E. coli* LPS, and Gregory McDonald for offering constructive comments.

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*Editor:* P. E. Orndorff