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Development of an In Vitro Model for Study of Non-O1 *Vibrio cholerae* Virulence Using Caco-2 Cells

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Non-O1 *Vibrio cholerae* strains have been reported as a causative agent of diarrhea throughout the world. We recently reported that non-O1 *V. cholerae* strains cause diarrhea in human volunteers. In this study we evaluated the virulence of three strains of non-O1 *V. cholerae* in a Caco-2 cell adherence assay by light and electron microscopy. A-5 is an environmental isolate which failed to colonize volunteers and did not cause diarrhea. It exhibited low numbers of organisms adherent to Caco-2 cells, leaving the microvilli intact. Strain 2076-79, isolated from a patient with diarrhea, colonized human volunteers without producing disease. It adhered to Caco-2 cells in moderate numbers without producing any damage to the microvilli. Strain NRT36S, a clinical isolate, colonized human volunteers and produced significant diarrheal disease. This strain adhered in very large numbers to Caco-2 cells and caused damage to the brush borders. Membrane-bound bacteria were also seen within the cytoplasm of these cells. Scanning electron microscopy confirmed the generalized adherence of NRT36S to the microvilli of Caco-2 cells. The three strains did not appear to compete with each other for binding sites on Caco-2 cells and were not adherent when assays were conducted at 4°C. Our results with strains A-5, 2076-79, and NRT36S correlate well with observations in human volunteer studies, suggesting that Caco-2 cells provide an appropriate in vitro system for further investigation of the pathogenesis of non-O1 *V. cholerae* gastroenteritis.

Vibrio cholerae strains in O group 1 are the causative agent of cholera. Recently, *V. cholerae* of O groups other than 1 (non-O1 *V. cholerae*) have been recognized as a cause of gastroenteritis in humans (19, 20, 21). Non-O1 strains have been isolated from patients with diarrhea throughout the world (1, 6, 7, 14, 20, 21). These organisms are part of the normal estuarine bacterial flora and have been isolated from the Atlantic, Pacific, and Gulf coasts in the United States (15, 16, 24). Human infection in the United States has generally been associated with eating raw oysters, particularly during warm, summer months when *Vibrio* counts in estuarine shellfish harvest areas are the highest (29). A variety of toxins have been proposed as possible virulence factors for non-O1 *V. cholerae* (2, 5, 12, 13, 18, 27, 30, 31). It has also been suggested that virulence is dependent on the ability of a non-O1 *V. cholerae* strain to colonize the intestine (28).

Recent volunteer studies have demonstrated that non-O1 *V. cholerae* strains can cause diarrhea (21a). Three non-O1 strains have now been administered to volunteers: strains 2076-79 and NRT36S, initially isolated from patients with diarrhea, and strain A-5, an environmental isolate. The two clinical isolates caused comparable colonization in volunteers (21a); however, only strain NRT36S caused disease (Table 1). The mechanisms by which these strains colonized the intestine remain to be determined. Fimbriae have been implicated in intestinal colonization (8, 10, 25, 27), and strain 2076-79was fimbriated; however, NRT36S was not. The factors responsible for the occurrence of diarrhea after ingestion of strain NRT36S (but not 2076-79) also remain to be determined.

The Caco-2 cell line, derived from a moderately differentiated human colonic adenocarcinoma (23), has recently been shown to be useful in the evaluation of adherence and invasion of bacterial pathogens (9, 17). In this study we infected Caco-2 cells with non-O1 *V. cholerae* strains A-5, 2076-79, and NRT36S. We were interested in determining whether adherence to Caco-2 cells correlated with human intestinal colonization and/or disease occurrence. We were also interested in assessing the utility of Caco-2 cells as an in vitro model for studying the pathogenesis of non-O1 *V. cholerae* infections.

MATERIALS AND METHODS

Bacterial strains. The three strains of non-O1 *V. cholerae* we studied were strain A-5, isolated from frozen shrimp in Japan (2); 2076-79, isolated from a Mississippi patient who had diarrhea after eating raw oysters (20); and NRT36S, from a patient with traveler's diarrhea in Tokyo (21a). These three strains were administered to human volunteers in a series of studies (21a). Characteristics of strains, as previously reported (21a) are shown in Table 1. For the current studies, strains were grown on colonization factor agar (CFA) containing 1% casamino acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract (Difco), 0.05% $MgSO_4$, 0.005% $MnCl_2$, and 2% agar (pH 7.4). Cultures from frozen stocks were incubated overnight at 37°C.

Antisera. Antisera against NRT36S and 2076-79 were raised in New Zealand White rabbits. Either 10^6, 10^7, or 10^8 CFU of bacteria in 1 ml of sterile saline was injected

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TABLE 1. Characterization of non-O1 *Vibrio cholerae* strains[a]

Characteristic	2076-79	A-5	NRT36S
Country where isolated	U.S.	Japan	Japan
Source	Stool, patient with diarrhea	Frozen shrimp	Stool, patient with diarrhea
Serotype (Smith)	O17	O31	O31
Tissue culture (cytotoxic response[b])			
Y-1 adrenal cells	16	64	8
CHO cells	16	64	8
Cholera toxin	−	−	−
El Tor hemolysin	+	+	+
Kanagawa hemolysin	−	−	−
Production of shigalike toxin (cell lysate)	+	−	−
Non-O1 heat-stable enterotoxin (NAG-ST)	−	+	+
Piliation (electron microscopy)	+	−	−
Response of volunteers			
Colonization	+	−	+
Diarrhea	−	−	+

[a] Data from Morris et al. (21a).
[b] Reported as reciprocal titer of highest dilution of culture supernatant that gave a positive response.

intravenously into rabbits at intervals of 10 days. The rabbits were boosted with 10^{10} CFU of bacteria per ml. Blood was collected 3 days after the final boost. Anti-NRT36S and anti-2076-79 hyperimmune sera were adsorbed to 2076-79 and NRT36S (heterologous bacterial strains), respectively, for 5 h at 4°C before use in the immunofluorescence experiments.

Cell culture. The Caco-2 cell line, which originated from a moderately differentiated human colonic adenocarcinoma (23), was generously donated by Philippe J. Sansonetti of the Service des Enterobactéries, Institut Pasteur, Paris, France. The cell line was cultured in minimal Eagle medium (MEM) with Earle salts and glutamine (Hazelton Biologics, Inc., Lenexa, Kan.) supplemented with 10% fetal bovine serum (FBS) (GIBCO Life Technologies, Inc., Grand Island, N.Y.), 1% nonessential amino acids, 1% sodium pyruvate (Flow Laboratories, Rockville, Md.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of fungizone (Whitaker M. A. Bioproducts, Walkersville, Md.) per ml. For the adherence assay, 5×10^5 Caco-2 cells were seeded into 12 ml of medium and distributed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.) containing 13-mm Thermanox tissue culture cover slips (Lab-Tek Division, Miles Laboratories, Inc., Naperville, Ill.) in each well. After incubation for 72 to 96 h at 37°C in a 10% CO_2–90% air atmosphere, the culture medium was changed to MEM with 2% FBS. Cell cultures were maintained with biweekly changes of the medium until brush border differentiation was complete at approximately 10 to 12 days postseeding.

Adherence assay. Each of the three strains of non-O1 *V. cholerae* was harvested in MEM without FBS or antibiotics from the CFA plates. The 12-day-old Caco-2 cell monolayers were washed three times with phosphate-buffered saline (PBS) and infected with bacteria. Bacteria were not washed to avoid loss or damage of any surface structures. After incubation at 37°C in 10% CO_2–90% air, monolayers were washed three times with PBS, fixed in methanol, and stained with Giemsa. The monolayers were examined by oil immersion light microscopy to determine adherence by each bacterial strain.

Adherence was noted after 15 min of incubation and reached its peak between 30 min and 1 h. For strains A-5 and 2076-79, patterns of adherence were not affected by incubation time (≥30 min) or by inoculum size (10^6 to 10^{10} CFU). For NRT36S at an inoculum of 10^6 CFU, a mean of ca. 25 bacteria adhered to each Caco-2 cell after 15 min of incubation. This number increased to ca. 50 bacteria per cell at 1 h, but there were many damaged cells in each microscopic field. When the infecting dose was increased to 10^8 CFU of NRT36S per ml, the degree of adherence was comparable to 25 bacteria per cell at 15 min, increasing to ca. 50 bacteria per cell at 30 min. There was no damage to cell membrane integrity at this point. At 1 h with an infecting dose of 10^8 CFU/ml, 10 to 15% of cells were damaged. The number of damaged cells increased to 75% by the end of 3 h, making the quantitation of bacteria difficult. Based on these observations, we conducted all assays with 10^8 CFU of bacteria per ml at 30 min of incubation.

The number of cells with adherent non-O1 *V. cholerae* and the number of adherent bacteria per cell were counted for 100 randomly chosen cells. The adherence assay was conducted three times, and the data were analyzed statistically.

The same adherence assay was repeated at 4°C for 30 min after chilling the bacterial suspension and cell cultures on ice for 15 min. Control inocula were maintained at 37 and 4°C and were titrated by plate counts to check whether there was any drop in counts of viable non-O1 *V. cholerae* after exposure to cold.

Immunofluorescence. Caco-2 cells were grown on chamber slides (Nunc Inc., Naperville, Ill.) as described under cell culture above. Monolayers were washed three times in PBS and infected with each of the non-O1 strains. After 30 min of incubation at 37°C, monolayers were thoroughly washed

TABLE 2. Adherence of non-O1 *Vibrio cholerae* strains to Caco-2 cells by light microscopy[a]

Strain	Mean % adherence-positive cells ± SD[b]	Mean no. of bacteria/cell[c]
A-5	66.33 ± 4.5	4.47
2076-79	86.0 ± 3.6	8.37
NRT36S	91.1 ± 2.6	50.24

[a] Assays were read after 30 min of incubation at 37°C.
[b] For A-5 versus 2076-79, $P < 0.04$; for A-5 versus NRT36S, $P < 0.01$; for 2076-79 versus NRT36S, $P < 0.19$.
[c] $P < 0.0001$ for A-5 versus 2076-79, A-5 versus NRT36S, and 2076-79 versus NRT36S.

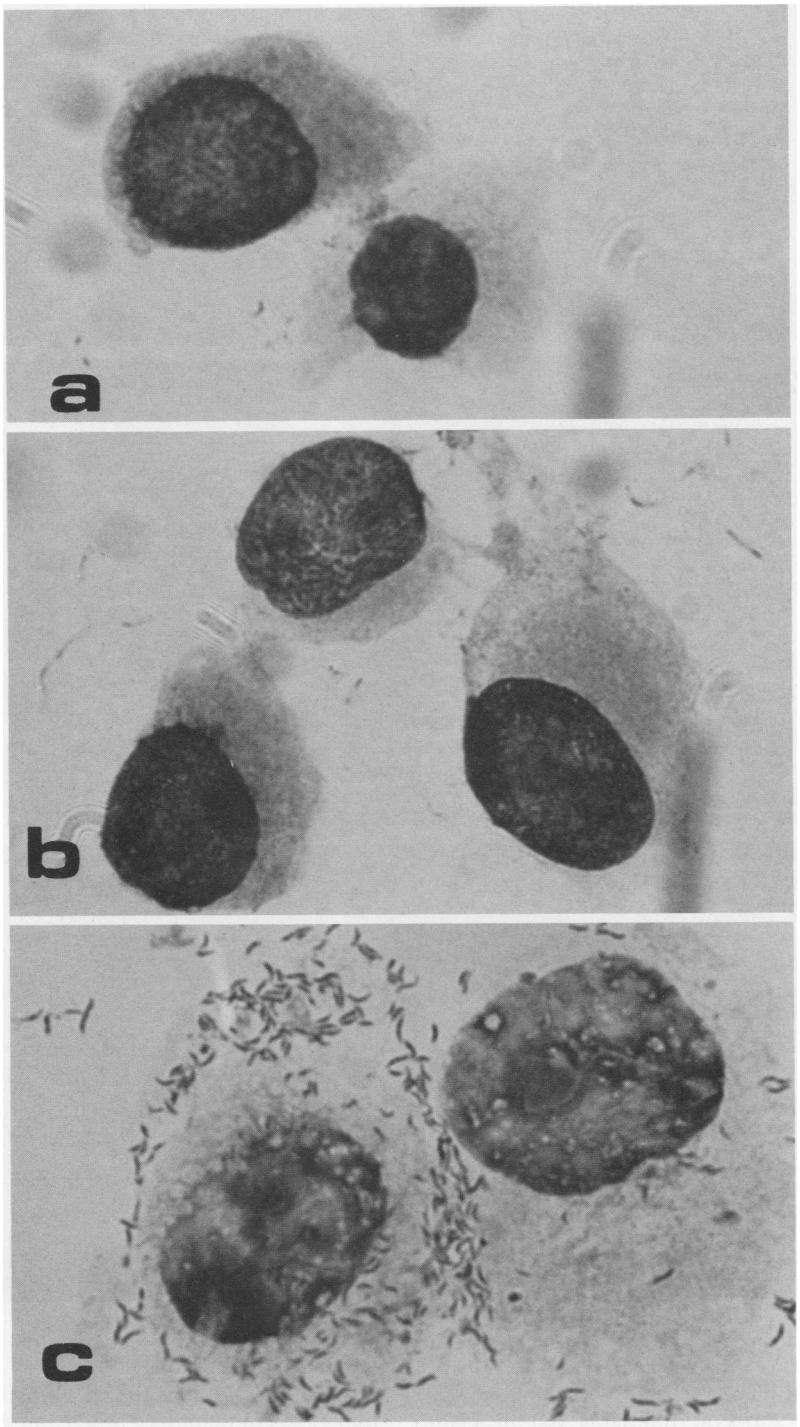
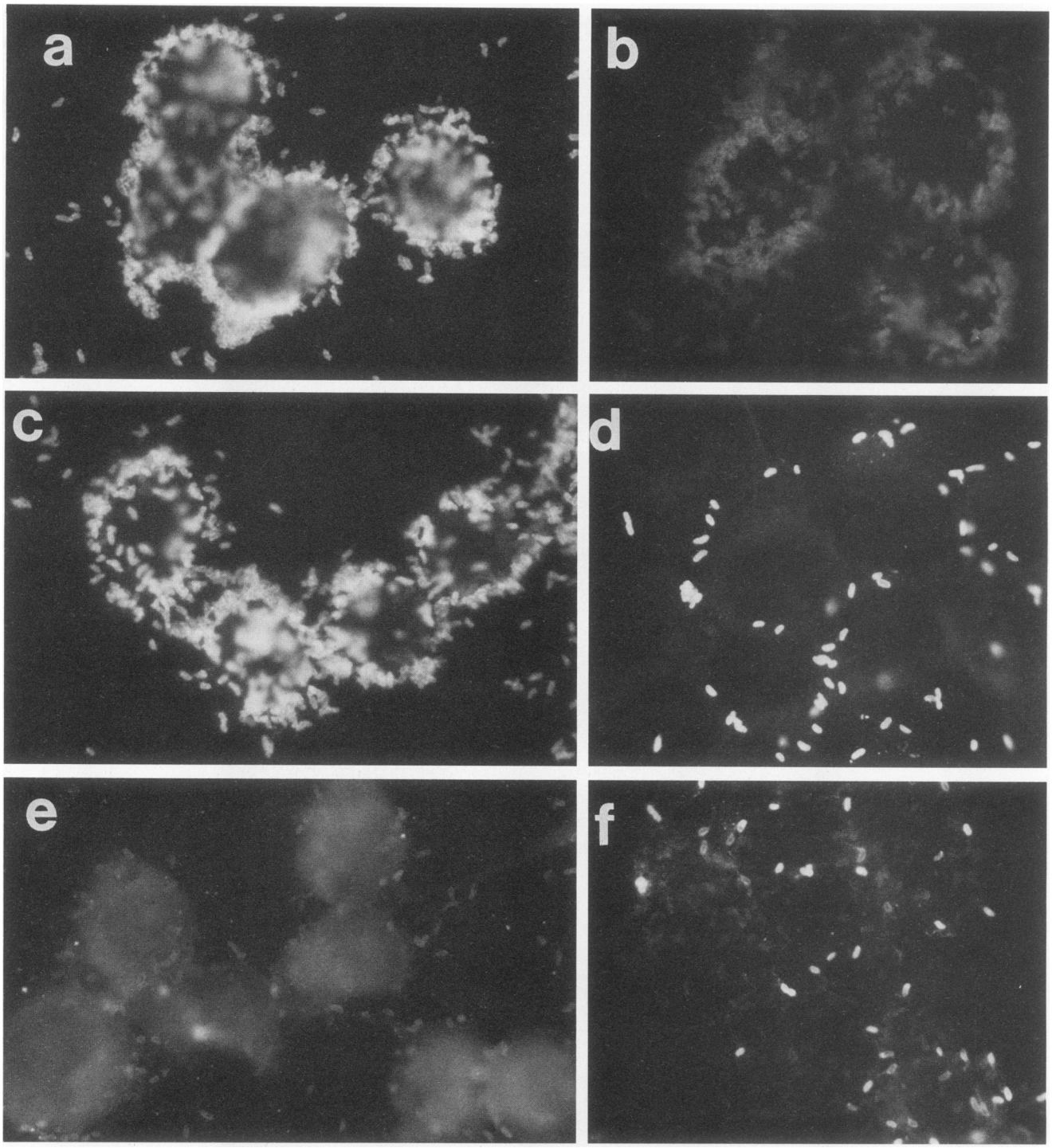


FIG. 1. Adherence pattern of non-O1 *V. cholerae* strains to Caco-2 cells by light microscopy. (a) Cells infected with strain A-5 show very few adherent bacteria. (b) Strain 2076-79, showing adherent bacteria in low numbers. (c) Remarkably high numbers of strain NRT36S showing generalized adherence to Caco-2 cells.



four times, and the slides were overlaid with homologous and heterologous rabbit antisera, diluted 1:100 in PBS. Incubation was continued for 30 min at 20°C to avoid phagocytic activity. Slides were washed four times in PBS and air dried at 20°C. Monolayers were then fixed in 100% methanol at −15°C for 1 min. After air drying, the monolayers were overlaid with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) diluted 1:50 in PBS and incubated for 20 min at 37°C. Finally the slides were washed four times in deionized water, air dried, and examined with a Zeiss fluorescence microscope under an oil immersion objective.

For the competitive inhibition study, double infection of the monolayers was conducted. After incubating the monolayers with one strain of bacteria for 30 min, a thorough washing in PBS was performed, and cells were infected with the second strain of bacteria and incubated for an additional

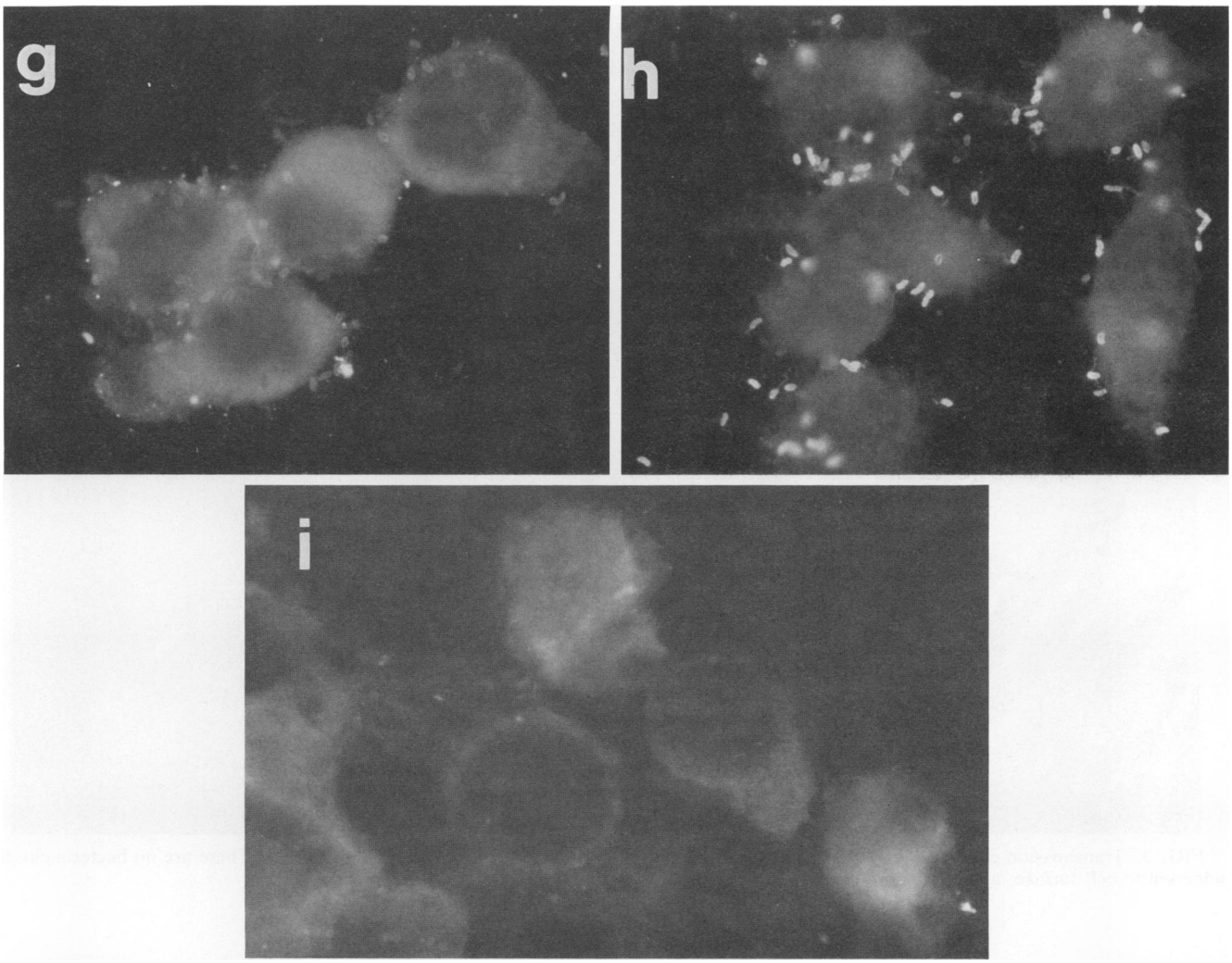


FIG. 2. Immunofluorescence pattern of non-O1 *V. cholerae* adherent to Caco-2 cells. (a) NRT36S with anti-NRT36S antiserum. (b) NRT36S with anti-2076-79 antiserum. (c) Cells infected with 2076-79, washed, reinfected with NRT36S, and treated with anti-NRT36S antiserum. (d) 2076-79 with anti-2076-79 antiserum. (e) 2076-79 with anti-NRT36S antiserum. (f) Cells infected with NRT36S, washed, reinfected with 2076-79, and then treated with anti-2076-79 antiserum. Note the brightly staining 2076-79 over a background of nonfluorescent NRT36S. (g) A-5 treated with anti-2076-79 antiserum. (h) Cells infected with A-5, washed, reinfected with 2076-79, and treated with anti-2076-79 antiserum. Note the binding of 2076-79 (bright fluorescence) over nonfluorescent A-5. (i) Control Caco-2 cells treated with anti-NRT36S antiserum and FITC conjugate.

30 min. This was followed by washing, incubation with antiserum to the second strain, and preparation for immunofluorescence as described above.

Transmission electron microscopy. Caco-2 cell monolayers in six-well plates were inoculated with 10^8 CFU of each strain of non-O1 *V. cholerae* per ml and incubated for 30 min to 3 h. The cells were washed vigorously three times in PBS and fixed with 2% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4, for 1 h. The cells were removed with a rubber policeman, washed twice with 0.2 M sodium cacodylate (pH 7.4) for 10 min, and stored overnight at 4°C in the cacodylate buffer. The tissues were postfixed in 1% OsO_4 in cacodylate buffer for 1 h. The postfixed cells were washed in cacodylate buffer, dehydrated in a graded series of ethanol (30 to 100%), and embedded in Epon by a rapid embedding technique (22). Thin sections were cut and placed on 300-mesh Formvar-carbon grids, stained with uranyl acetate and lead citrate, and examined under a JEOL 100 B transmission electron microscope operating at an accelerating voltage of 60 kV.

Scanning electron microscopy. Monolayers grown on 13-mm cover slips were infected with 10^8 CFU of each strain of non-O1 *V. cholerae* per ml for 30 min. Cover slips were washed three times and fixed in 2% glutaraldehyde in cacodylate buffer. The specimen was then dried in a critical-point drier, gold coated, and examined in an AMR 1000 scanning electron microscope.

Statistical analysis. The number of cells showing adherence was calculated per 100 cells in each adherence assay, and the mean and standard deviation of adherence-positive cells were calculated for the three experiments. The numbers of adherent organisms were also analyzed further. The unit of observation was the mean of the number of organisms adherent to 100 cells, including the nonadhering cells. The mean and standard error were calculated on the basis of multiple experiments under identical conditions. Random-

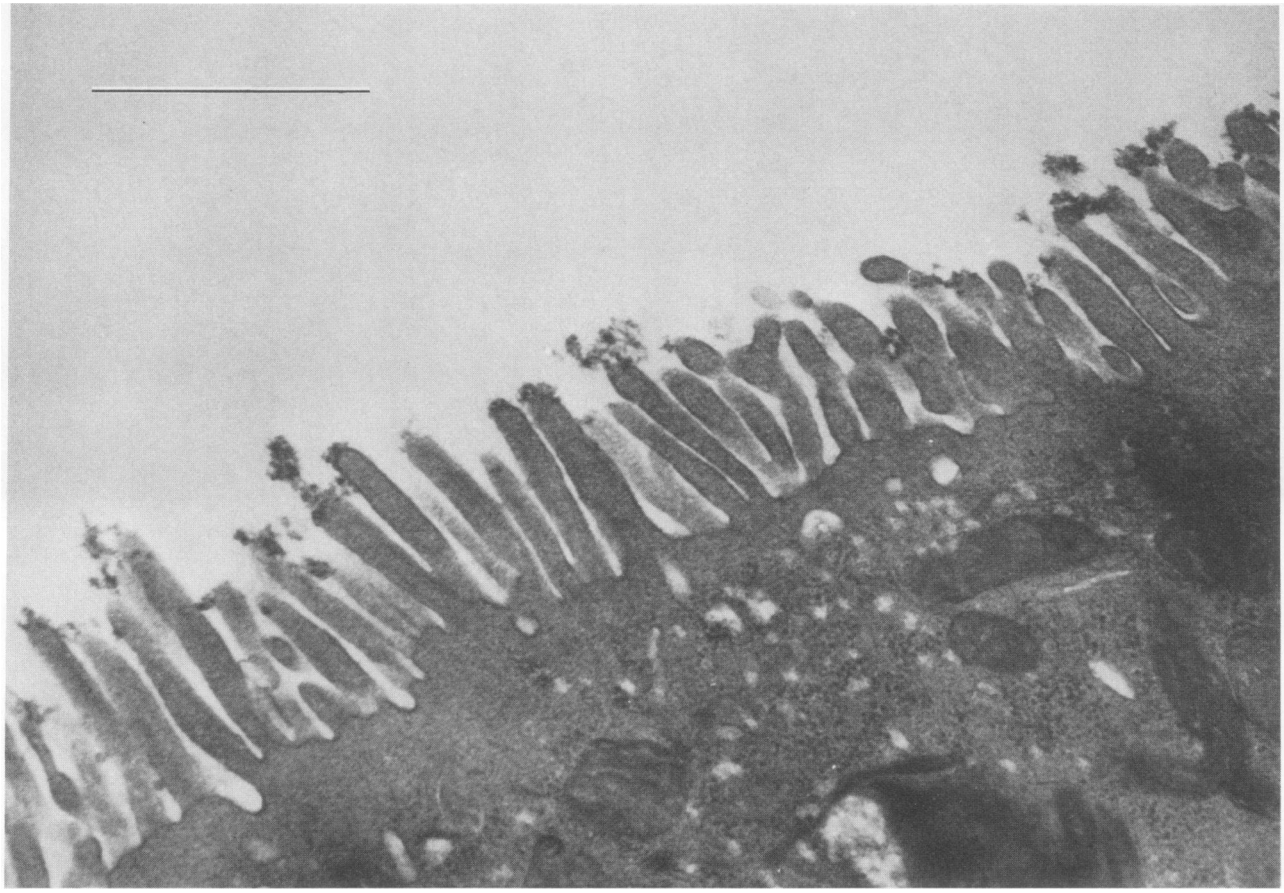


FIG. 3. Transmission electron micrograph of Caco-2 cell infected with strain A-5 of non-O1 *V. cholerae*. There are no bacteria closely adherent to cell surface, and the brush border is intact. Bar, 1 μm.

ized-complete-blocks analysis of variance was performed to compare the means of adherence of the three different strains and also the number of adherence-positive cells for each strain.

RESULTS

Adherence of non-O1 *V. cholerae* to Caco-2 cells. In the Caco-2 assay, the three strains of non-O1 *V. cholerae* demonstrated different patterns of adherence. In studies with strain A-5, bacteria were adherent to approximately 66% of Caco-2 cells. The rate of adherence increased with 2076-79 ($P < 0.04$), with bacteria adherent to 86% of cells. With strain NRT36S, 91% of cells showed positive adherence ($P < 0.01$) (Table 1). The adherence of A-5, 2076-79, and NRT36S strains to Caco-2 cells is demonstrated by light microscopy photomicrographs in Fig. 1. The mean number of organisms of each strain adherent to Caco-2 cells is shown in Table 2. Strain 2076-79 showed a significant increase in the number of adherent organisms compared with strain A-5 ($P < 0.0001$). Remarkably higher numbers (approximately 50 bacteria per cell) of strain NRT36S were adherent compared with both 2076-79 and A-5 ($P < 0.0001$).

At 4°C, all three strains of non-O1 *V. cholerae* exhibited two to three adherent bacteria per cell, which was considered background binding. The viability of the non-O1 strains, as determined by plate counts, was not affected by cooling to 4°C.

Competitive inhibition study. Figure 2 shows the immunofluorescence pattern of non-O1 *V. cholerae* adherent to Caco-2 cells. Strain NRT36S (Fig. 2a) and 2076-79 (Fig. 2d) showed bright fluorescence when treated with homologous antisera. The same strains showed a low background staining with heterologous antisera (Fig. 2b and e). Figure 2c shows cells infected with 2076-79, washed, reinfected with NRT36S, and then treated with anti-NRT36S antiserum. The degree of binding of NRT36S (brightly staining bacteria) appeared to be unchanged; however, because of the large numbers of NRT36S, it was difficult to comment on the presence or absence of 2076-79. In contrast, when cells were first infected with NRT36S, washed, reinfected with 2076-79, and then treated with anti-2076-79 antiserum (Fig. 2f), the brightly staining 2076-79 (unchanged in number compared with 2076-79 alone) could be clearly seen over a background of nonfluorescent NRT36S. A-5 did not react with anti-2076-79 antiserum (Fig. 2g), and, as expected, there was no difference in the binding pattern of 2076-79 after A-5 adherence (Fig. 2h). Figure 2i shows control Caco-2 cells treated with anti-NRT36S antiserum and FITC conjugate.

Electron microscopy. Electron microscopy was performed to analyze the nature of adherence of *V. cholerae* to Caco-2

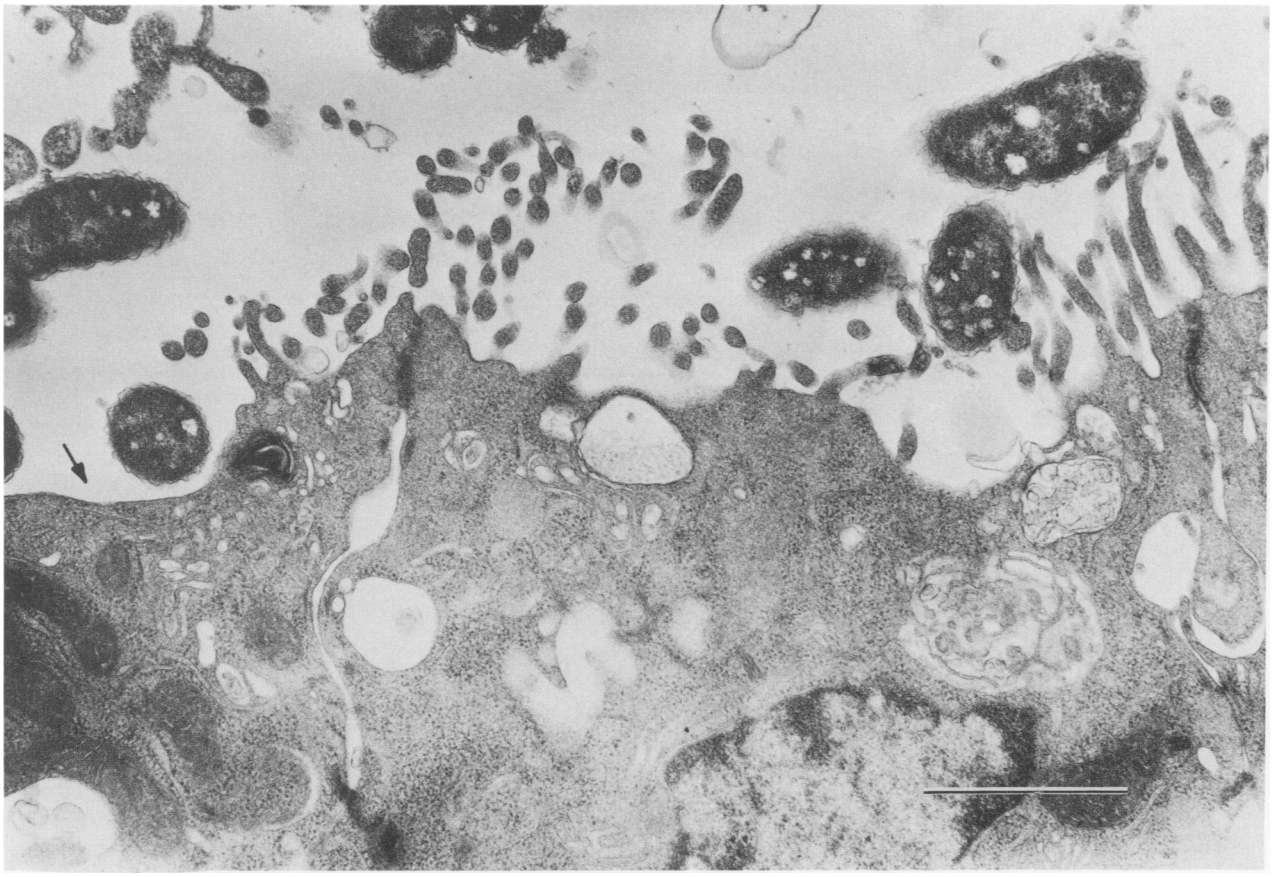


FIG. 4. Transmission electron micrograph of Caco-2 cell infected with strain NRT36S of *V. cholerae*. There are bacteria adherent to the brush border, with damage in patchy areas (arrow). Bar, 1 μm.

cells at the ultrastructural level (Fig. 3 to 5). With strain A-5, no bacteria were found closely attached to the Caco-2 cells, and the brush border was intact (Fig. 3). In some electron microscopic fields, small numbers of A-5 were seen in the vicinity of Caco-2 cells, but bacteria were not intimately attached to the brush border. The 2076-79 preparation showed a small number of organisms adherent to Caco-2 cells without any damage to the brush border. As expected from the light microscopic studies, strain NRT36S exhibited many bacteria adherent to each cell, and there was damage to the brush border in patchy areas (Fig. 4) after 30 min of incubation. Invasion by NRT36S was evidenced by membrane-bound bacteria within the cytoplasm of Caco-2 cells. The brush borders were grossly damaged in these cells with invaded NRT36S organisms (Fig. 5). However, damage to the brush borders was also clearly apparent in cells with no evidence of bacterial invasion. After 3 h of incubation with NRT36S, most of the Caco-2 cells showed damage to the cell membrane integrity, with multiple bacteria in and around the cytoplasm. Scanning electron microscopy of Caco-2 cells infected with NRT36S showed large numbers of bacteria adherent to microvilli of Caco-2 cells (Fig. 6).

DISCUSSION

The Caco-2 cells used in this study are derived from a human colon carcinoma and differentiate into enterocyte-like cells in the absence of inducers. The differentiation occurs at late confluency, when the cells express typical brush border microvilli and tight junctions. These cells functionally express high levels of alkaline phosphatase and sucrase isomaltase, considered typical of human small intestinal cells (23). In our preparations, brush borders were present in the 12-day-old Caco-2 cell cultures used for the adherence assay. Recently these cells were characterized for their physiological and biochemical properties and used as a model involving investigations in human enterocytes (3, 11). Caco-2 cells have also been used to study adherence, invasion, and intracellular growth of bacterial pathogens such as *Escherichia coli* (17) and *Listeria monocytogenes* (9).

Non-O1 *V. cholerae* cause a variety of symptoms, including mild to moderate diarrhea and septicemia, and also asymptomatic infection (20, 26). In this study we evaluated an in vitro assay to assess the adherence and invasion properties of strains of non-O1 *V. cholerae* in an attempt to predict clinical illness. Differences in adherence were observed between A-5 and 2076-79 (noncolonizing and colonizing strains in humans, respectively). However, the most striking difference was between NRT36S (which both colonized and caused disease in humans) and A-5 and 2076-79. The mechanisms responsible for these differences remain to be determined. Strain 2076-79 is fimbriated, and it is possible that fimbriae mediated adherence to Caco-2 cells (8, 10, 25, 27). However, NRT36S, which was more adherent to Caco-2

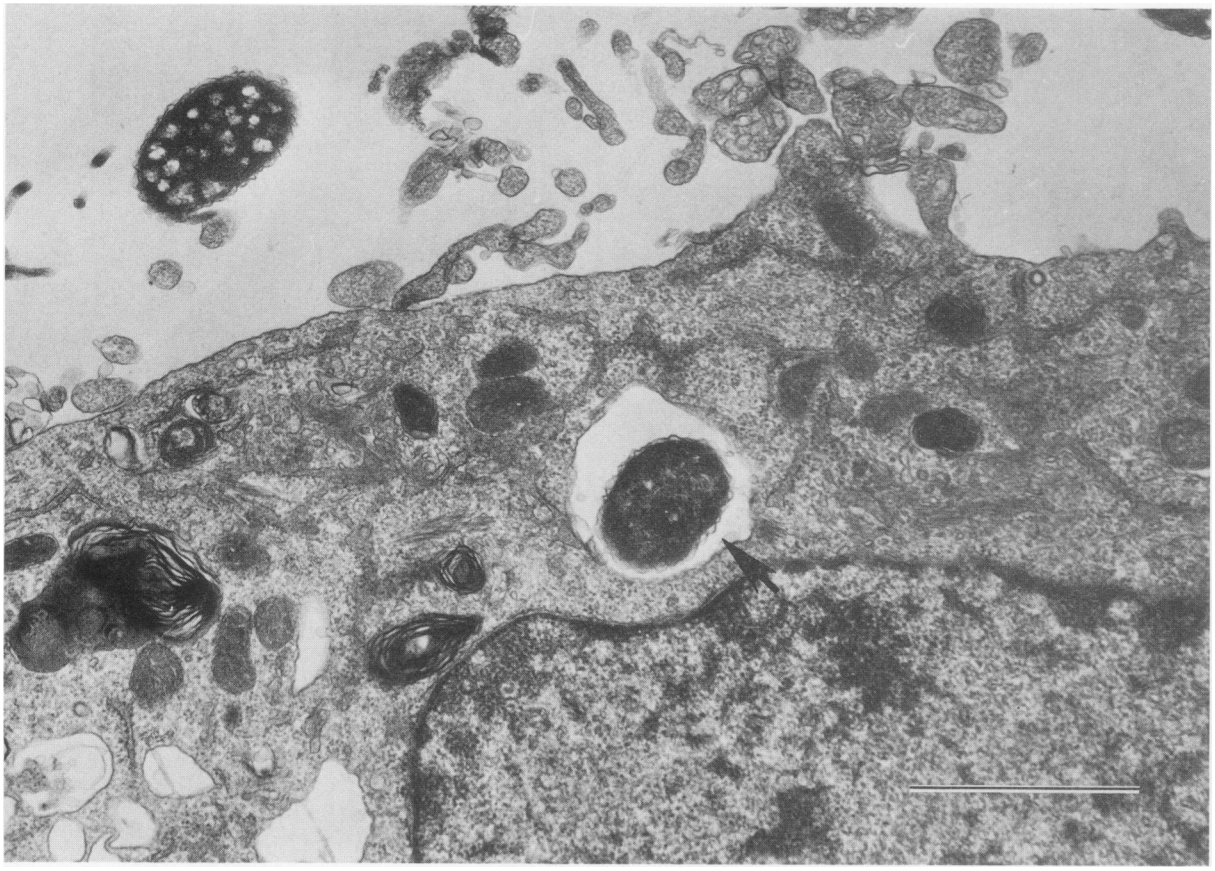


FIG. 5. Transmission electron micrograph of Caco-2 cell infected with strain NRT36S of *V. cholerae*. Note the membrane-bound bacteria in the cytoplasm (arrow) and the grossly damaged microvillous cell surface. Bar, 1 μm.

cells than 2076-79, has not been found to produce fimbriae. As one possibility, strains of NRT36S may have nonfimbrial adhesive factors, similar to those described for certain strains of *E. coli* (4).

The same adherence assay conducted at 4°C did not show any adherence with any of the three strains, providing at least some evidence that the bacteria (and/or the Caco-2 cells) have to be metabolically active to adhere to Caco-2 cells. We also looked at the possibility of competitive inhibition of adherence of one strain by the other. The immunofluorescence data suggest that the two adherent strains NRT36S and 2076-79 do not compete for binding sites on Caco-2 cells. Strain 2076-79 also adhered in similar numbers to Caco-2 cells over already adherent A-5. A-5 and NRT36S belong to the same serogroup, and A-5 exhibited a strong fluorescence pattern with anti-NRT36S antiserum. Hence it was not possible to determine whether these two strains compete for binding to Caco-2 cells.

NRT36S caused brush border damage and appeared to invade Caco-2 cells. NRT36S carries the gene for the El Tor hemolysin, which causes cytotoxic activity (13, 18), and produces a heat-stable enterotoxin (designated NAG-ST) (2, 12). However, both 2076-79 and A-5 also have the El Tor hemolysin genes, and A-5 produces NAG-ST. In previous studies we have noted that cytotoxicity in CHO and Y-1 adrenal cells was actually less pronounced with culture supernatants of NRT36S than with the supernatants from the other two strains (Table 1). Cell invasion by NRT36S is a novel finding that has not been demonstrated for other *V. cholerae* strains.

The implications of the Caco-2 cell invasion in the pathogenesis of diarrheal disease by NRT36S are unclear. Non-O1 *V. cholerae* has been reported to cause bloody diarrhea (20), suggesting that invasion of epithelial cells occurs in vivo. However, the volunteers who ingested NRT36S had neither gross nor occult blood in their stools (21a). Further studies are needed to define the mechanisms responsible for the in vitro effects observed in our Caco-2 system and to elucidate the relationships among adherence, tissue damage, invasion, and clinical disease.

In this study we restricted our analysis to these three strains because they are the only non-O1 *V. cholerae* strains that have been administered to volunteers and consequently are the only strains for which we have confirmed data on virulence in humans. Given the differences in patterns of adherence that we observed, our Caco-2 system appears to provide a good in vitro model for investigating mechanisms of adherence and invasion of non-O1 *V. cholerae*. While further studies will be necessary to determine whether the results seen with these three strains are applicable to all non-O1 *V. cholerae*, this or a similar assay may ultimately provide a means of distinguishing potentially pathogenic from nonpathogenic non-O1 *V. cholerae* isolates.

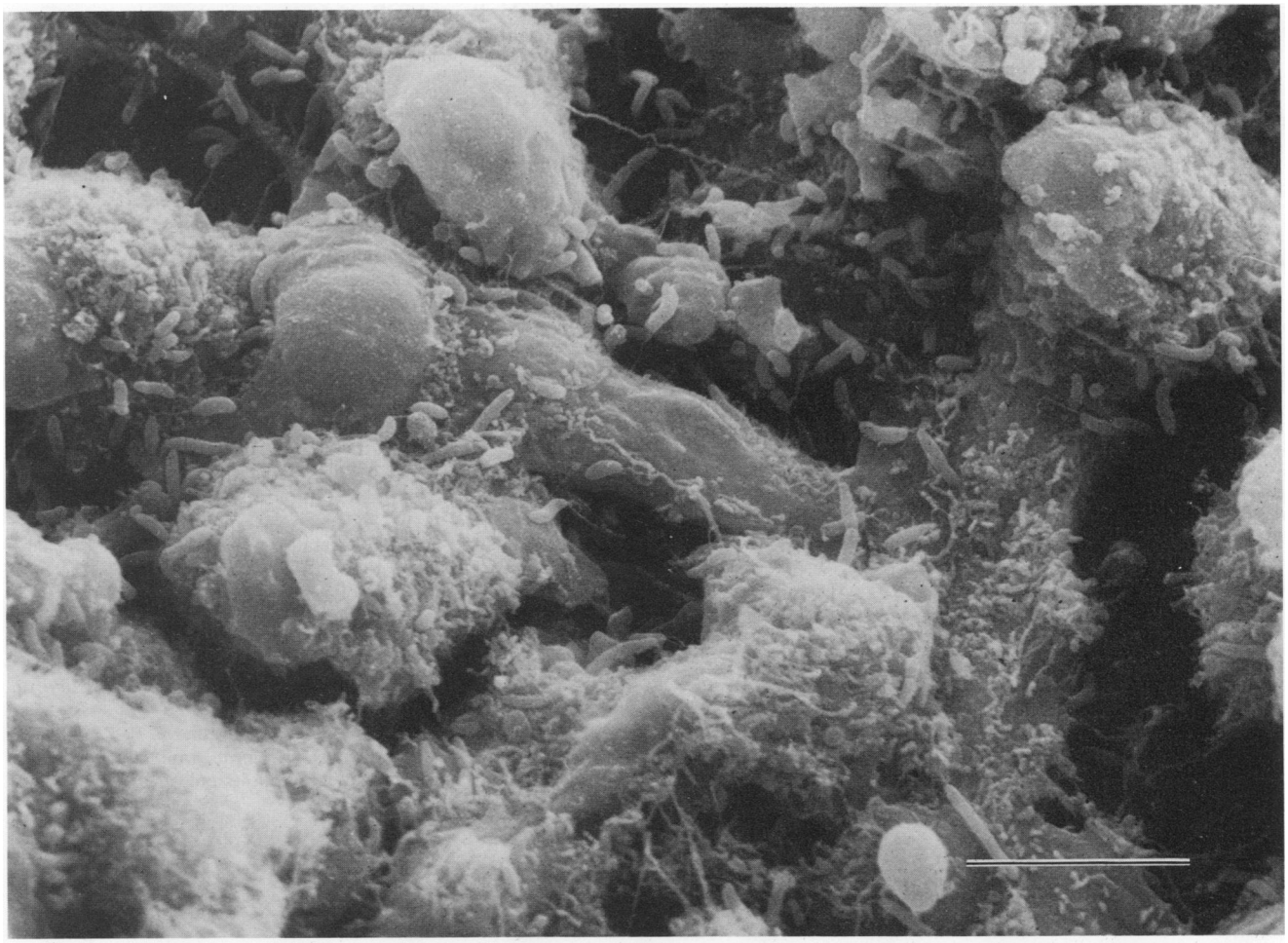


FIG. 6. Scanning electron micrograph of Caco-2 cell infected with strain NRT36S of *V. cholerae*. The entire microvillous surface of the cell is loaded with bacteria, with patchy damage. Bar, 5 μm.

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