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Electron Microscopic Study of *Vibrio cholerae* O1 Adherence to the Mucus Coat and Villus Surface in the Human Small Intestine

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***Vibrio cholerae* O1, irrespective of the biotype or serotype, adhered to and was entrapped in the mucus coat covering the mucosal surface of isolated human ileal segments. The evidence for such mucus coat adherence was obtained by treatment of the ileal segments with 10% Formalin. In any case, adherence to the mucus coat was much more prominent than adherence to the epithelial cell surface of the small intestinal villi. Mucus coat adherence was affected by sugars and by the growth phase of the bacterial culture and was diminished by the heating of *V. cholerae* O1. We conclude that the small intestinal mucus coat is a primary adherence target for *V. cholerae* O1 in human infection and that the cell-associated hemagglutinin of *V. cholerae* O1 plays a role, at least in part, in adherence.**

In *Vibrio cholerae* O1 infection, the initial, essential steps in the development of cholera, followed by oral infection, are understood to be the adherence and subsequent proliferation of the pathogen on the free surface (with numerous, very small projections, called microvilli, which constitute brush borders) of the absorptive cells (10, 11, 13). The absorptive cells are located at the single epithelial cell layer of the villi, which are also numerous tiny projections, reaching from the small intestinal mucosa into the lumen. The intestinal villi are covered with mucus, which is a gel mainly consisting of large, viscous, and polydispersed glycoproteins (mucins) (4–6, 9). It is produced and secreted by the goblet cells, another type of epithelial cells, located at the surface layer of the villi. Previous investigations by other laboratories have shown that *V. cholerae* O1 cells occasionally become entrapped in the mucus coat, without showing evidence of adherence (10, 13). Moreover, it has been shown that a lag period exists before the appearance on (attachment to) the surface of the villi of *V. cholerae* O1 injected into the lumen, indicating the possibility that the mucus coat may act as a barrier against bacterial penetration (13). Those previous observations on the mucus entrapment of *V. cholerae* O1 were made in a rabbit model. In this communication, we demonstrate that *V. cholerae* O1, irrespective of the biotype or serotype, adheres to the mucus coat of the human small intestine and that adherence to the mucus coat is more extensive than adherence to the villus surface of the human small intestine.

Specimens of the human small intestine used in this study were terminal segments of the small intestine excised from patients (aged 28 to 54 years) with ascending colon cancer at Juntendo Hospital. The specimens were donated by Toshiki Kamano, First Department of Surgery, Juntendo University. The small intestinal segments were then opened, and the bulk of the tissue at the serosal side (from the tunica serosa to the submucosa) was cut off and discarded. The remaining mucosal side of the small intestine (from the muscularis mucosae to the mucosal epithelium with mucus) was washed several times with fresh, cold (4°C) phosphate-buffered saline, pH 7.4. The mucus did not dissolve in the buffer and thus remained over the mucosal surface. A slice (1 cm^2) of the washed small intestinal mucosa (with mucus) was immediately used for adherence experiments. In some experiments, the washed small intestinal mucosa (with mucus) was fixed with 10% (vol/vol) Formalin in KRT (10) consisting of 7.5 g of NaCl, 0.383 g of KCl, 0.318 g of $MgSO_4 \cdot 7H_2O$, and 0.305 g of $CaCl_2$ in 10 mM Tris hydrochloride, pH 7.4, by the method described previously (10, 12) and maintained at 4°C. Prior to adherence experiments, the Formalin-fixed mucosa (with mucus) was washed in KRT, pH 8.0, and part of the mucus coat covering the mucosal surface was carefully removed with soft tissue papers. The Formalin-fixed mucosa (with mucus) was then cut into 0.5-cm^2 pieces and washed with cold (4°C) KRT, pH 7.4, with two buffer changes (500 ml each) for 3 h.

The bacterial strains used are summarized in Table 1. For the adherence assay, the bacteria were grown on CFA agar (3) consisting of 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract (Difco), 0.005% $MgSO_4$, 0.0005% $MnCl_2$, and 2% agar (pH 7.4) for 3 to 20 h at 37°C. The bacterial cells grown on the surface of the CFA agar plates were suspended in KRT, pH 7.4, at a concentration of 600 Klett units (measured in a Klett-Summerson colorimeter with a red filter). Portions of the bacterial suspensions were also tested for cell-associated hemagglutinin (cHA). For this, twofold serial dilutions of the bacterial suspensions (300 Klett units) were made with KRT, pH 7.4, and 100-μl samples were mixed with 100 μl of 3% human (group A) erythrocytes in a 24-well multidish plate (diameter of each well, 15 mm; A/S Nunc, Roskilde, Denmark). After 20 min at room temperature (ca. 22°C), hemagglutination (HA) titers (the highest dilution to yield positive results) were determined by using a light microscope. The results roughly corresponded to those of HA on glass slides. In experiments in which the effect of sugar on the HA reaction was examined, 3% human erythrocytes containing 1 or 2% (wt/vol) sugar (L-fucose or D-mannose) were used instead of 3% human erythrocytes alone. For experiments in which the effect of EDTA was examined, bacterial cells were diluted with KRT, pH 7.4, containing 10 mM EDTA (pH 7.4), and then mixed with 3% human erythrocytes. HA titers thus obtained are shown in Table 1 (data are representative of three trials).

The conditions for bacterial adherence to the human intestinal mucosa (with mucus) were those developed for the study of *V. cholerae* O1 adherence to rabbit mucosa (10, 12).

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TABLE 1. Bacterial strains and characteristics of cHAs

V. cholerae O1 strain[a]	Incubation time (h) at 37°C required for growth	HA titer with addition[b]:				Place and yr of isolation and source of strain
		None	L-Fucose	D-Mannose	EDTA	
Biotype classical						
Serotype Inaba						
X-23222 (CI3)	3	1:64	<1:1	1:64	<1:1	Bangladesh 1982, Y. Takeda
	20	<1:1	<1:1	<1:1	<1:1	
Serotype Ogawa						
VC-203 (CO12)	3	1:64	1:16	1:64	1:2	Japan 1946, M. Ohashi
59-4121 (CO11)	3	1:128	1:128	1:128	1:32	This laboratory
Biotype E1 Tor						
Serotype Inaba						
CVC84-2 (EI3)	3	1:8	1:8	1:2	1:8	Nepal 1984, K. Koiwai
Serotype Ogawa						
TVC48 (EO8)	3	1:32	1:16	1:32	1:16	Japan 1981, M. Ohashi

[a] Designations used in this study are shown in parentheses.
[b] Added at 0.5 or 1% (wt/vol) (L-fucose and D-mannose) or 5 mM (EDTA).

A piece of human intestinal mucosa (with mucus; fixed or unfixed with Formalin), prepared as above, was immersed into 1.5 to 2 ml of bacterial suspension at 600 Klett units (in KRT, pH 7.4), followed by incubation for 10 min (or 30 min, where indicated) at 28°C. When the effect of the sugars on adherence was examined, the bacterial cells and a slice of the mucosa (with mucus) were separately preincubated for 5 min in KRT, pH 7.4, containing 1% (wt/vol) L-fucose or 1% (wt/vol) D-mannose, and then a slice of the mucosa (with mucus) was moved into the sugar-containing bacterial suspensions, followed by incubation for 10 min at 28°C, as above. The effect of EDTA (5 mM) on adherence was also tested in a similar way. The mucosa (with mucus) was immediately washed four times in KRT, pH 7.4, and fixed in a KRT (pH 7.4) solution containing 2.5% (vol/vol) glutaraldehyde and 2% (wt/vol) tannic acid for 2 h at room temperature and subsequently postfixed in 1% (wt/vol) osmium tetroxide for 2 h (or overnight) at 4°C. The fixed samples were dehydrated with acetone and critical-point dried. The samples were then coated with gold-palladium and analyzed by scanning electron microscopy.

The human ileal mucosa was deeply covered with a mucus coat, as shown in Fig. 1A. Most of the villi in the ileal segments tested possessed many goblet cells at the very tip as well as the side. When looked at from the lumen side, the goblet cells lacking the brush border were observed as a hole, producing mucus, surrounded by numerous brush borders of the absorptive cells (Fig. 1B). This was very different from the villi found in the human jejunal segments tested, in that the jejunal villi possessed only a limited number of mucus-producing holes of the goblet cells on the surface.

When a slice of the ileal mucosa (with mucus), as analyzed in Fig. 1A, was exposed to *V. cholerae* O1 CI3 (biotype classical:serotype Inaba), grown for 3 h at 37°C and manifesting a high level of cHA (Table 1), it was found that many of the CI3 cells were entrapped in the mucus coat (Fig. 2A and B). Heating of the CI3 cells (a high cHA producer) to 60°C for 30 min, by which the cHA activity was completely inactivated, resulted in a marked decrease in the entrapment of CI3 cells in the mucus coat (Fig. 2C). The weak entrapment in the mucus coat was also observed with CI3 cells which were grown for 20 h at 37°C and manifested no detectable cHA (Table 1), as shown in Fig. 2D.

Next, we determined the ability of *V. cholerae* O1 to adhere to the mucus coat. For this, the human ileal mucosa with a mucus coat was fixed with 10% Formalin and then

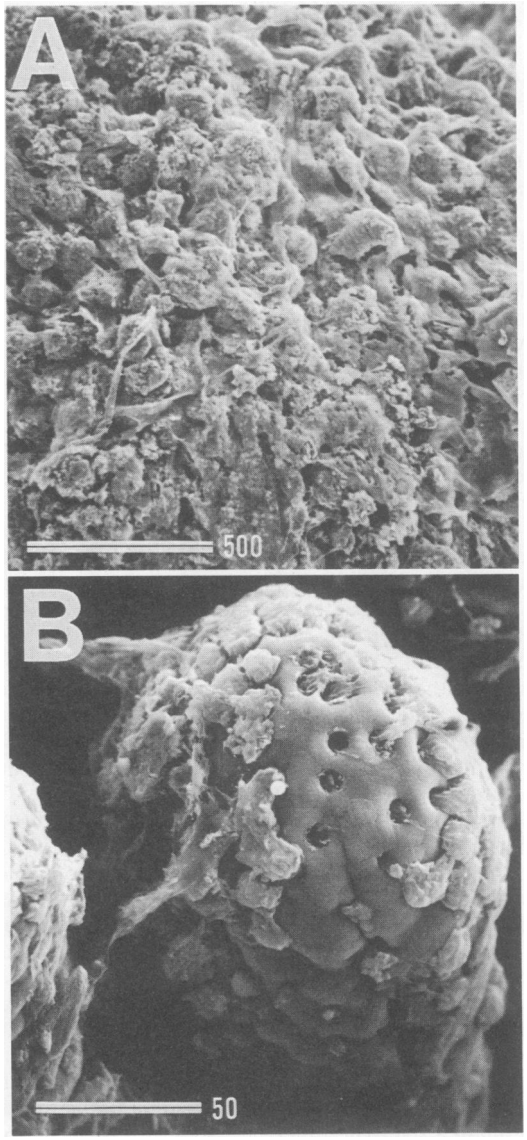


FIG. 1. Scanning electron micrograph of the control human ileal mucosa covered with deep mucus coat (A) and of the human ileal villi possessing the goblet cells producing mucus (B). Bars, 500 μm (A); 50 μm (B).

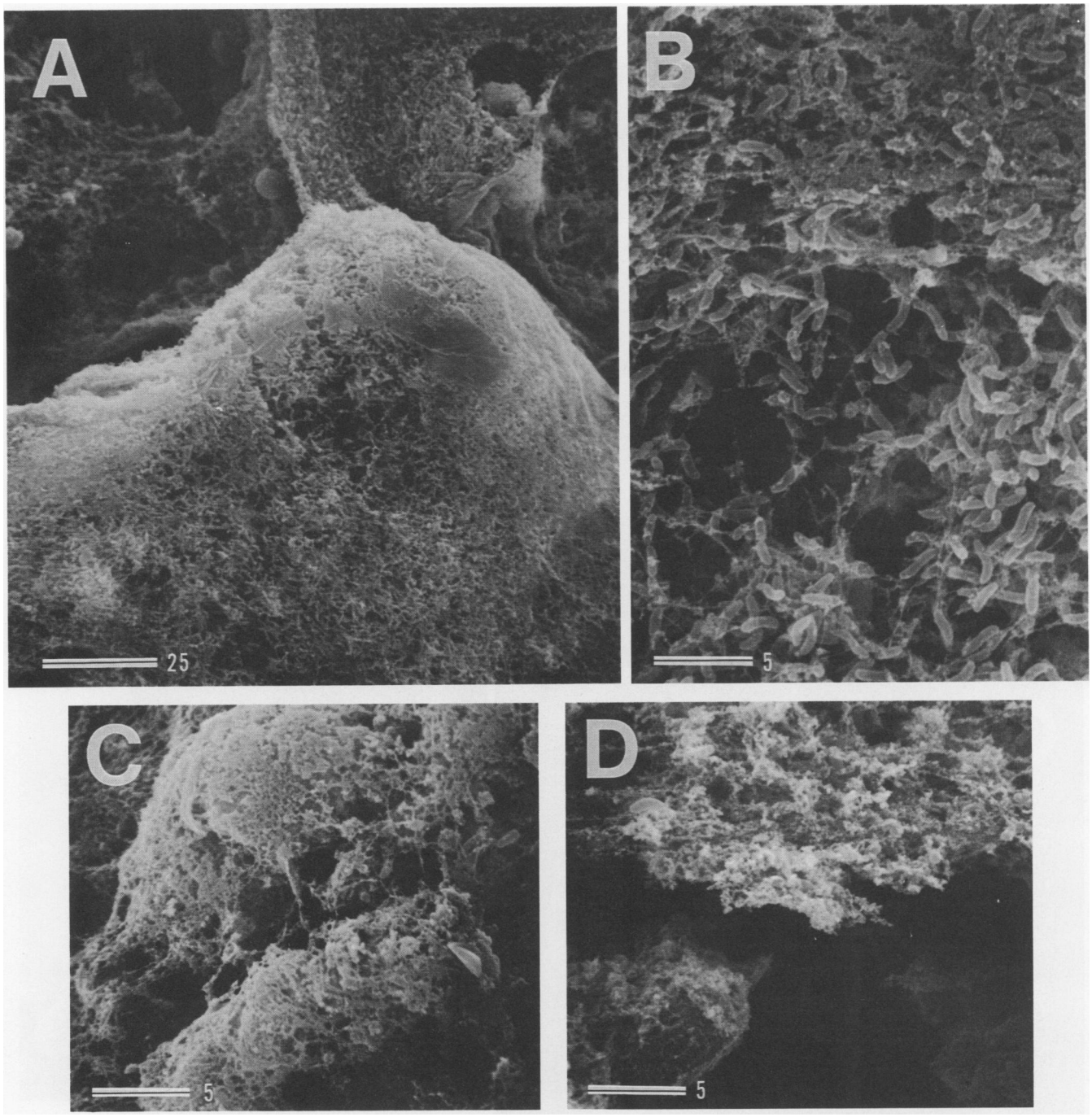
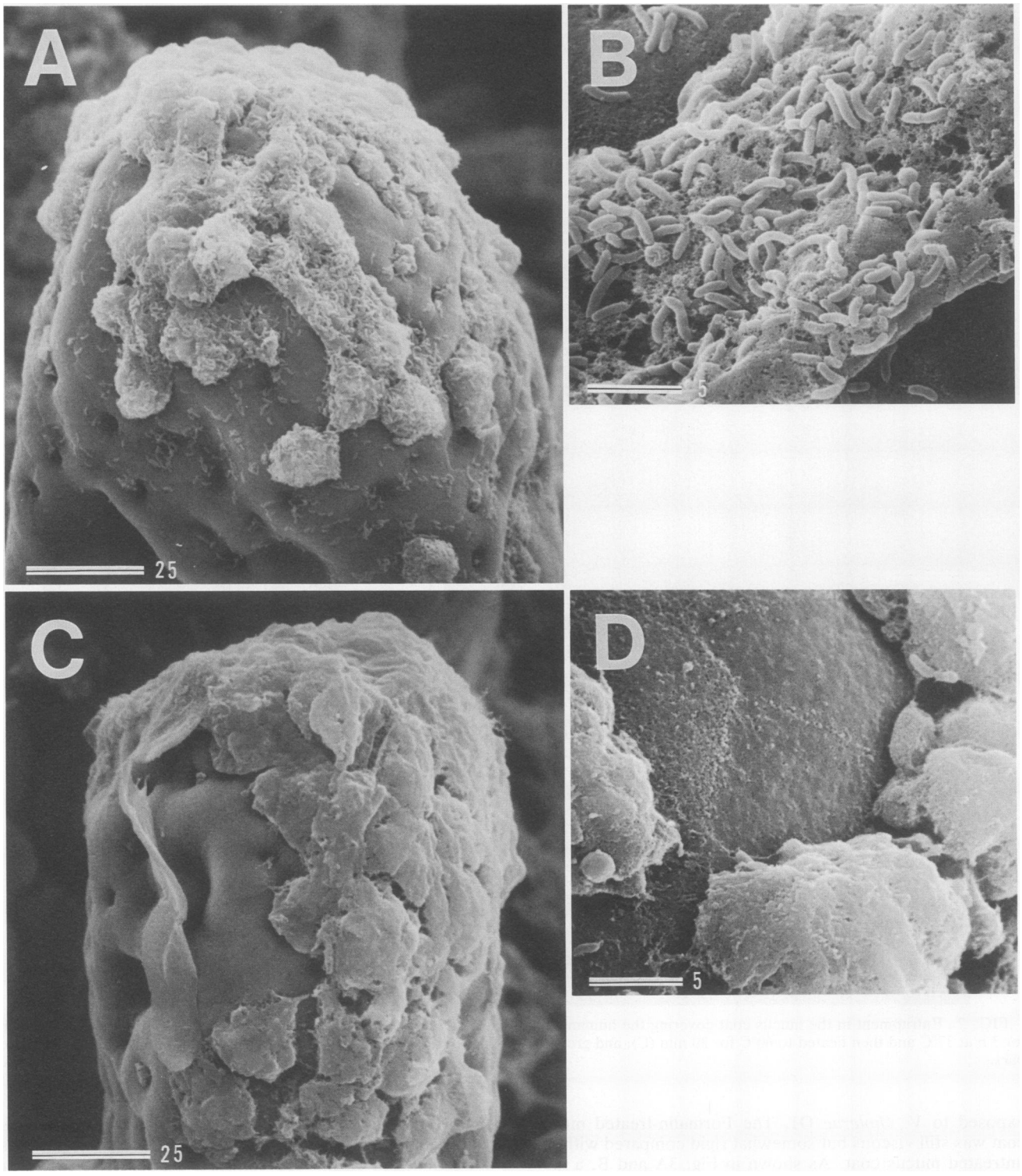


FIG. 2. Entrapment in the mucus coat covering the human ileal mucosa of *V. cholerae* O1 CI3 grown for 3 h at 37°C (A and B), grown for 3 h at 37°C and then heated to 60°C for 30 min (C), and grown for 20 h at 37°C (D). Numbers represent lengths (in micrometers) of scale bars.

exposed to *V. cholerae* O1. The Formalin-treated mucus coat was still viscous but somewhat rigid compared with the untreated mucus coat. As shown in Fig. 3A and B, a high cHA producer variant of the CI3 strain grown for 3 h at 37°C strikingly adhered to the surface of the mucus coat which spread over the villi. Such adherence was prominent compared with adherence to the epithelial surface of the villi; this phenomenon was also observed when the mucosa and CI3 cells were incubated for a longer period (30 min instead of 10 min). Because of the rigidity of the Formalin-treated mucus coat, penetration of *V. cholerae* O1 into the mucus coat was not significant. In marked contrast, a poor cHA producer variant of the CI3 strain, grown for 20 h at 37°C, barely adhered to the surface of the Formalin-treated mucus coat (Fig. 3C and D). Since the HA reaction due to the cHA of the CI3 strain was sensitive to L-fucose and resistant to D-mannose (Table 1), the effect of the sugars on adherence of the CI3 cells (a high-cHA producer variant) to the Formalin-


A
25
B
5
C
25
D
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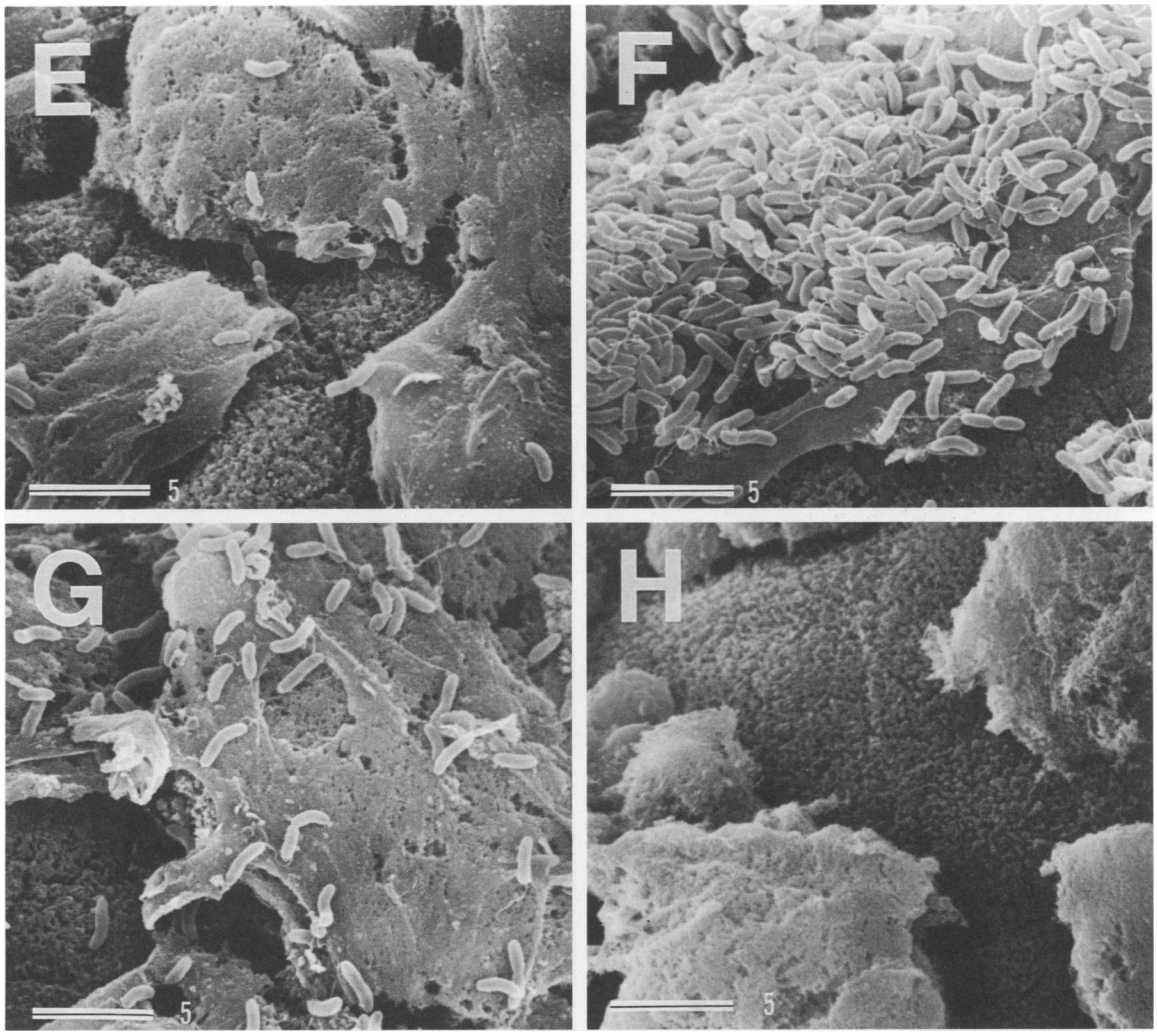

FIG. 3. Adherence to the Formalin-treated mucus coat on the human ileal villi of *V. cholerae* O1 CI3 grown for 3 h at 37°C (A and B), grown for 20 h at 37°C (C and D), grown for 3 h at 37°C and then analyzed in the presence of 1% L-fucose (E), 1% D-mannose (F), or 5 mM EDTA (G), and grown for 3 h at 37°C and then heated to 60°C for 30 min (H). Numbers represent lengths (in micrometers) of scale bars.

treated mucus coat was tested. In accordance with the sugar inhibition pattern observed in the HA reaction, L-fucose at a concentration of 1% had a marked (but not complete) inhibitory effect (Fig. 3E; compare with Fig. 3B), while D-mannose at the same concentration had a stimulatory effect (Fig. 3F; compare with Fig. 3B). There existed, however, the contradiction that at lower concentrations, e.g., 0.5%, L-fucose still completely inhibited the HA reaction (Table 1), whereas it only slightly inhibited adherence of the CI3 cells to the mucus coat (data not shown). A similar contradiction was observed with the effect of EDTA (5 mM); it completely inhibited the HA reaction (Table 1), but only partially inhibited adherence of the CI3 cells to the mucus coat (Fig. 3G; compare with Fig. 3B), confirming an earlier observation (7) that calcium (Ca^{2+}) was required for the L-fucose-sensitive HA reaction, whereas omission of Ca^{2+} from the suspending buffer did not affect *V. cholerae* O1 adherence to isolated rabbit intestinal mucosa. As shown in Fig. 3H, when a high-cHA producer variant of the CI3 strain was heated to 60°C for 30 min (and thus lacked cHA activity), the CI3 cells lost the ability to adhere to the surface of the mucus coat (compare with Fig. 3B).

The observation that adherence to the human ileal mucus coat was prominent compared with adherence to the epithelial surface of the human ileal villi was also obtained with other cHA-producing strains (grown for 3 h at 37°C, Table 1) of *V. cholerae* O1 belonging to biotype classical:serotype Ogawa (Fig. 4A and B), biotype E1 Tor:serotype Inaba (Fig. 4C), or biotype E1 Tor:serotype Ogawa (Fig. 4D). Again, heating of bacteria to 60°C for 30 min to inactivate cHA activity resulted in a marked loss of the ability to adhere to the human ileal mucus coat in any case, as shown in Fig. 3H for the CI3 strain. L-Fucose at a concentration of 1% had no marked inhibitory effect on the mucus adherence of strains CO11, EI3, and EO8. D-Mannose at a concentration of 1% also had no marked inhibitory effect on the mucus adherence.

This study clearly demonstrated that *V. cholerae* O1 can adhere to the mucus coat covering the human ileal mucosa. The fact that *V. cholerae* O1 adhered to Formalin-treated human intestinal mucus coat agreed with the earlier notion (1) that some bacterial enteropathogens, including *V. cholerae* O1, recognize sugar residues of receptors with their adhesins. The data for the CI3 strain indicated that the cHA

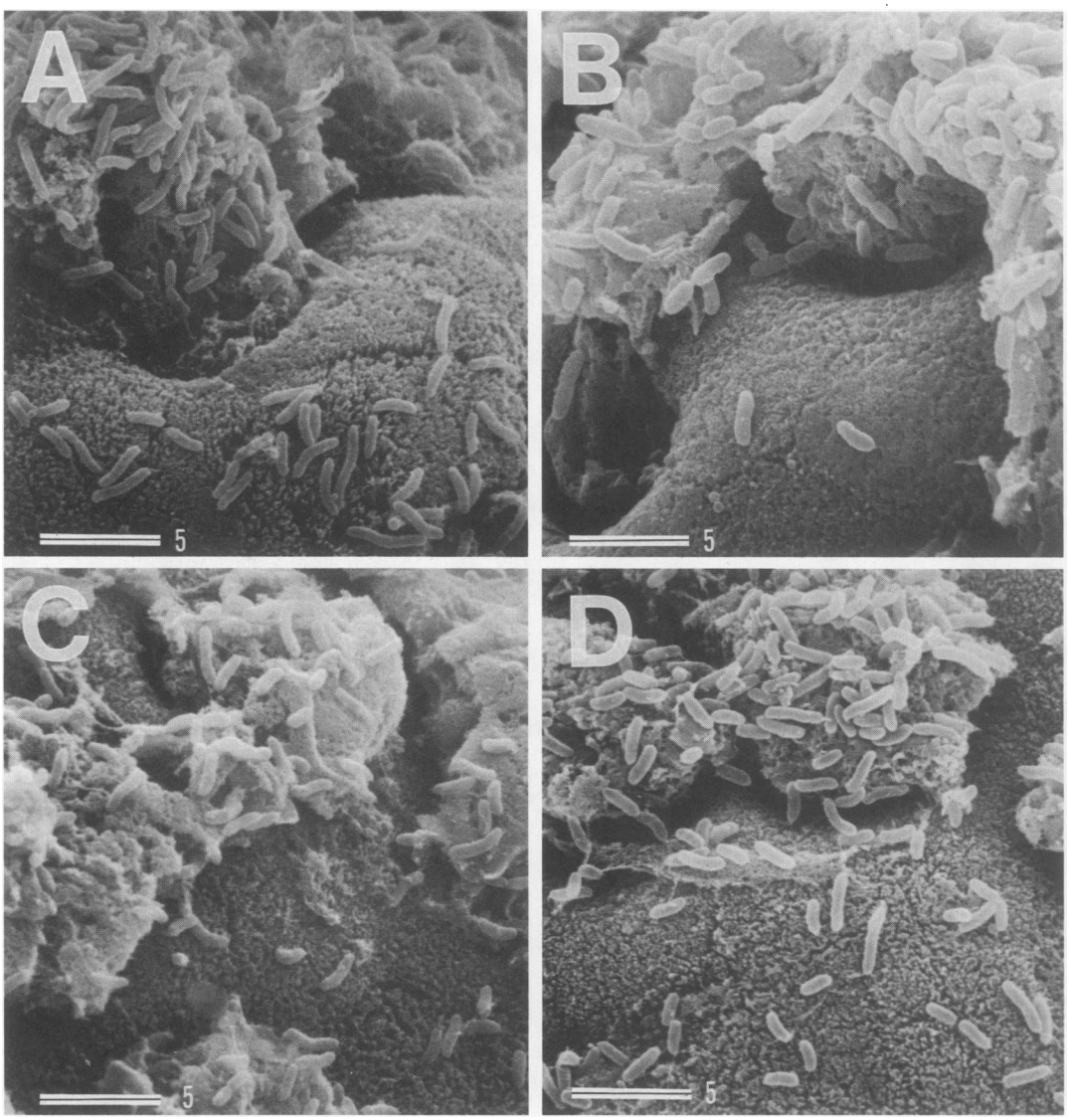

FIG. 4. Adherence to the Formalin-treated mucus coat present on the human ileal villi of *V. cholerae* O1 strains CO11 (A), CO12 (B), EI3 (C), and EO8 (D). Numbers represent lengths (in micrometers) of scale bars.

plays a role, at least in part, in adherence. It is conceivable that the cHA of the CI3 strain recognizes L-fucose-containing sugar residues of the mucin or glycocalyx of the human small intestinal mucosa. Perhaps the most important finding of this study was that *V. cholerae* O1 adhered to the mucus coat more markedly than to the epithelial surface of the villi. Based on this fact, it was concluded that the mucus coat covering the small intestinal mucosa is a primary adherence target for *V. cholerae* O1 in human infection. The question then arises of how *V. cholerae* O1, being so tightly attached to the mucus gel, can move to the absorptive cell microvilli, which are presumed to be the adherence site of *V. cholerae* O1 (10–13). One possibility under investigation is that the small intestinal mucus coat is also the site for the proliferation of *V. cholerae* O1 and that the *V. cholerae* O1 multiplied there then move to the absorptive cell microvilli. Another possibility is that "adhesins" of *V. cholerae* O1 are not saturated with "receptors" in the mucus coat, so that *V. cholerae* O1 can move down through the mucus coat (10) and adhere to the absorptive cell microvilli. Still another possibility is that gaps exist in the mucus coat (14) and that *V. cholerae* O1 can reach the absorptive cell microvilli through these gaps.

Earlier work (8) has shown that *V. cholerae* O1 belonging to biotype classical:serotype Inaba expresses L-fucose-sensitive cHA transiently in the early log phase in liquid cultures. This study on the CI3 strain confirmed that it also occurs on CFA agar (Table 1). In addition, we have shown that most *V. cholerae* O1 strains, irrespective of biotype or serotype, best produce cHA as early as ca. 3 h of incubation at 37°C (on CFA agar) and that the in vitro adherence of *V. cholerae* O1 to the absorptive cell microvilli on the human jejunal or ileal villi roughly correlates with its cHA activity (T. Yamamoto, T. Kamano, M. Uchimura, M. Iwanaga, and T. Yokota, submitted for publication).

Pili of *V. cholerae* O1 have recently been characterized and noted from the viewpoint of possible adhesins of *V. cholerae* O1 (2, 15). We also confirmed the piliation of the CI3 strain grown on CFA agar. However, such piliation was observed more extensively at ~20 h of incubation at 37°C

(on CFA agar), where no detectable cHA was produced (Table 1) and very low levels of mucus adherence were observed (Fig. 2D and 3C and D) than at ~3 h of incubation at 37°C (on CFA agar), where levels of cHA as well as mucus adherence were markedly high (Fig. 2A and B and 3A and B). Thus, the piliation of *V. cholerae* O1 (CI3 strain) did not seem to correlate simply with the adherence or cHA activities detected (Yamamoto et al., submitted).

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