

## Adherence Targets of *Vibrio parahaemolyticus* in Human Small Intestines

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**Formalin-fixed human small intestinal mucosa with mucus coating, villi, and lymphoid follicle epithelium at the mucosal surface was used to test the adherence sites of clinically isolated (Kanagawa phenomenon-positive) strains of *Vibrio parahaemolyticus*. *V. parahaemolyticus* strains grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C possessed various levels of cell-associated hemagglutinins (HAs) which were detected with human or guinea pig erythrocytes. The observed adherence abilities of *V. parahaemolyticus* strains to human small intestinal mucosa correlated roughly with the HA levels of the strains. Under the test conditions, ileal lymphoid follicle epithelium (especially M cells) provided the best adherence target for *V. parahaemolyticus*. Adherence to villus absorptive cells or to mucus coating was observed at lower levels. In addition, all 3-h-grown *V. parahaemolyticus* strains tested produced high levels of HAs as detected with rabbit erythrocytes. The strains were all strikingly motile. In contrast, *V. parahaemolyticus* strains grown on CFA agar (supplemented with 3% NaCl) for ca. 20 h at 37°C had much lower levels of HAs, adherence abilities, and motility. In contrast to the above observations, piliation of *V. parahaemolyticus* was more extensive at ca. 20 h of incubation at 37°C than at ca. 3 h of incubation at 37°C. The remarkable ability of *V. parahaemolyticus* to adhere to lymphoid follicle epithelium was also confirmed by using rabbit small intestinal mucosa.**

*Vibrio parahaemolyticus* exists in estuaries and marine environments with seasonal variation in its incidence (2, 8, 9, 13, 16, 24). In humans, it causes gastroenteritis via contaminated seafood (2, 8, 9, 13, 24). Most isolates from patients tested manifest erythrocyte hemolysis on Wagatsuma agar (Kanagawa phenomenon positive). In contrast, most isolates from the marine environment are Kanagawa phenomenon negative (18, 25, 29). The Kanagawa phenomenon-associated hemolysis is due to the thermostable direct hemolysin produced by the bacteria (20, 21, 26-28).

Several earlier works have demonstrated adherence abilities of *V. parahaemolyticus*. Kaneko and Colwell (15) found that *V. parahaemolyticus* could associate with chitin or chitin-bearing copepods. Carruthers (6) reported that Kanagawa phenomenon-positive *V. parahaemolyticus* adhered to tissue-cultured human epithelial cell lines (HeLa or fetal intestinal cells) better than Kanagawa phenomenon-negative *V. parahaemolyticus*. Joseph et al. (14) showed that a Kanagawa phenomenon-negative clinical isolate of *V. parahaemolyticus* adhered to the villus surface in rabbit ileum and that the same strain colonizing in the ileum possessed pililike appendages. Levett and Daniel (17) reported the adherence to isolated rabbit brush border membranes of a Kanagawa phenomenon-positive clinical isolate of *V. parahaemolyticus*. Gingras and Howard (10) demonstrated that *V. parahaemolyticus* adhered to tissue-cultured human epithelial cell lines (intestine 407 and Flow 11000 cells) irrespective of the Kanagawa phenomenon. In this study, we demonstrated that *V. parahaemolyticus* Kanagawa phenomenon-positive strains, isolated from patients, produced cell-associated hemagglutinins (HAs) after as little as ca. 3 h at 37°C. We also demonstrated that those *V. parahaemolyticus* strains possessed the striking ability to adhere to lymphoid follicle epithelium, including M cells (22, 23), rather than to villus epithelium, in an in vitro adherence model system using Formalin-fixed human or rabbit small intestines.

### MATERIALS AND METHODS

**Bacteria, media, and bacterial growth.** *V. parahaemolyticus* strains used were isolated in 1982 through 1988 in Juntendo Hospital (Tokyo) from patients with gastroenteritis. For bacterial growth, we used colonization factor antigen (CFA) agar (7) supplemented with 3% NaCl, which consisted of 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract (Difco), 0.005% MgSO<sub>4</sub>, 0.0005% MnCl<sub>2</sub>, 3% NaCl, and 2% agar (pH 7.4). It was followed by incubation for 2 to 20 h at 37°C. Wagatsuma agar (Eiken, Tokyo) was kindly provided by K. Ohta. Nutrient broth (Eiken) with 3% NaCl was used as a liquid medium.

**Kanagawa phenomenon test.** One loopful (5 µl) of bacterial cultures, grown overnight at 37°C in nutrient broth with 3% NaCl, was streaked on the surface of Wagatsuma agar (containing 5% washed human erythrocytes), and the plates were incubated for 24 h at 37°C. Hemolysis around or below the colonies was evaluated.

**HA assay.** To test HA production, bacterial cells grown on the surface of CFA agar (supplemented with 3% NaCl) plates were suspended in KRT (12), consisting of 7.5 g of NaCl, 0.385 g of KCl, 0.318 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, and 0.305 g of CaCl<sub>2</sub> in 10 mM Tris hydrochloride (pH 7.4), to a concentration of 600 Klett units (measured in a Klett-Summerson colorimeter with a red filter). Twofold serial dilutions were then made with KRT, and 100-µl samples were mixed with 100 µl of 3% erythrocytes in a 24-well multidish plate (diameter of each well, 15 mm; A/S Nunc, Roskilde, Denmark). After the plates stood for 20 to 90 min at room temperature (ca. 22°C), HA titers were determined with a light microscope (24-well plate method; 30, 31). The effect of sugar on the HA reaction was examined in the same way, except that 3% erythrocytes containing 1% (wt/vol) sugar (L-fucose or D-mannose) were used instead of 3% erythrocytes alone. When the effect of EDTA or ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) on the HA reaction was tested, bacterial cells were diluted with KRT containing 10 mM EDTA (pH 7.4) or 10 mM

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EGTA (pH 7.4) and then mixed with 3% erythrocytes as described above. For measurement of HA activities, 96-well microtiter plates (U-well; SH-T96U; Terumo, Tokyo, Japan) were also used (microtiter plate method), and 25- $\mu$ l samples were mixed with 25  $\mu$ l of erythrocytes as described previously (12, 30).

**Preparation of small-intestine specimens.** Rabbit ileal specimens were prepared as described by Nakasone and Iwanaga (19). The ileum was excised from an adult rabbit (Japanese White) weighing 2.5 kg, washed with cold (4°C) phosphate-buffered saline (pH 7.4), and fixed with 10% (vol/vol) Formalin in KRT (12).

Specimens of the human small intestine used in this study were terminal ileum or jejunum excised from patients aged 28, 44, or 50 years with ascending colon cancer at Juntendo Hospital, and were prepared as described previously (30–32). The small-intestine segments were immediately opened, and the mucosal side of the small intestine (from the muscularis mucosae to the mucosal epithelium with mucus) was saved and washed several times with cold (4°C) phosphate-buffered saline. The mucosa was then fixed with 10% Formalin (in KRT) and maintained at 4°C. Prior to adherence experiments, the Formalin-fixed mucosa was cut into 0.5-cm<sup>2</sup> pieces and washed with cold (4°C) KRT with two buffer changes (500 ml each) for 3 h. Mucus covering the mucosal surface was carefully removed with soft tissue paper in some experiments.

**Adherence test.** Bacteria used were grown on CFA agar (supplemented with 3% NaCl) for 2 to 20 h at 37°C. Adherence experiments were conducted essentially by the method for *Vibrio cholerae* adherence (12, 19, 30, 31). Pieces of human or rabbit intestine samples prepared as described above were immersed in 1.5 to 2 ml of bacterial suspension at 600 Klett units in KRT, followed by incubation for 10 min (30 min where indicated) at 28°C. The intestine samples were immediately washed four times in KRT, fixed in a KRT 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.

**Scanning electron microscopy.** The fixed intestine samples were dehydrated with acetone and critical-point dried. The samples were then coated with gold-palladium and analyzed by scanning electron microscopy. In experiments in which adherence levels were determined, 30 or 60 observation fields obtained at a magnification of  $\times 4,000$  (23 by 28  $\mu$ m) were randomly chosen and photographed in every area tested (epithelial surface of villi and lymphoid follicles), and the number of bacteria was recorded for each sample. The average number of bacteria per electron-microscopic field (photograph) constituted the adherence index.

**Transmission electron microscopy.** Bacterial cells grown on CFA agar (supplemented with 3% NaCl) plates for 2 to 20 h at 37°C were suspended in saline. One drop of the bacterial suspension was applied to each of several carbon-coated collodion grid screens (3-mm diameter) for 60 s. The adherent bacteria on the screens were then negatively stained with 2% (wt/vol) uranyl acetate for 90 s. The stained grids were subjected to analysis with a transmission electron microscope.

## RESULTS

**HA production of CFA agar supplemented with 3% NaCl.** Ten clinically isolated (Kanagawa phenomenon-positive) strains of *V. parahaemolyticus* (Table 1) were tested for HA

TABLE 1. Cell-associated HA levels of *V. parahaemolyticus* strains grown on CFA (supplemented with 3% NaCl) agar for 3 or 20 h at 37°C

Strain	Erythrocytes used	HA titer <sup>a</sup> after bacterial incubation at 37°C for:				
		3 h, with addition in assay				20 h
		None	L-Fucose	D-Mannose	EDTA	
1013	Human <sup>b</sup>	1:1	1:1	<1:1	<1:1	<1:1
	Guinea pig <sup>b</sup>	1:4	1:4	1:1	<1:1	<1:1
	Rabbit <sup>c</sup>	1:64	1:64	1:64	1:16	1:16 <sup>d</sup>
1014	Human	1:2	1:2	<1:1	<1:1	<1:1
	Guinea pig	1:8	1:8	1:2	<1:1	<1:1
	Rabbit	1:64	1:64	1:64	1:64	1:8 <sup>d</sup>
1015	Human	1:2	1:2	<1:1	<1:1	<1:1
	Guinea pig	1:8	1:8	1:2	<1:1	<1:1
	Rabbit	1:128	1:128	1:128	1:64	1:8 <sup>d</sup>
100A	Human	1:2	1:2	<1:1	<1:1	<1:1
	Guinea pig	1:8	1:8	1:1	<1:1	<1:1
	Rabbit	1:128	1:128	1:128	1:64	1:8 <sup>d</sup>
100B	Human	1:2	1:2	<1:1	<1:1	<1:1
	Guinea pig	1:4	1:4	1:1	<1:1	<1:1
	Rabbit	1:128	1:128	1:128	1:64	1:16 <sup>d</sup>
1001	Human	<1:1	<1:1	<1:1	<1:1	<1:1
	Guinea pig	<1:1	ND <sup>e</sup>	ND	ND	<1:1
	Rabbit	1:64	1:64	1:64	1:8	1:16 <sup>d</sup>
1006	Human	<1:1	<1:1	<1:1	<1:1	<1:1
	Guinea pig	1:4	1:4	<1:1	<1:1	<1:1
	Rabbit	1:32	1:32	1:32	1:16	1:2 <sup>d</sup>
1002	Human	<1:1	<1:1	<1:1	<1:1	<1:1
	Guinea pig	<1:1	ND	ND	ND	<1:1
	Rabbit	1:64	1:64	1:64	1:32	1:16 <sup>d</sup>
1007	Human	<1:1	<1:1	<1:1	<1:1	<1:1
	Guinea pig	<1:1	ND	ND	ND	<1:1
	Rabbit	1:128	1:128	1:128	1:64	1:8 <sup>d</sup>
1016	Human	<1:1	<1:1	<1:1	<1:1	<1:1
	Guinea pig	<1:1	ND	ND	ND	<1:1
	Rabbit	1:64	1:64	1:64	1:8	1:16 <sup>d</sup>

<sup>a</sup> Data (HA titers) indicate the highest dilution which yielded positive results by the 24-well plate method and are representative of at least three trials. L-Fucose and D-mannose were added to 0.5% (wt/vol); EDTA was added to 5 mM (the same results were obtained when EGTA at 5 mM was used instead of EDTA).

<sup>b</sup> Human and guinea pigs HAs were resistant to L-fucose and (moderately) sensitive to D-mannose; HA titers were scored after 30 min of incubation at room temperature.

<sup>c</sup> Rabbit HAs were resistant to both L-fucose and D-mannose; HA titers were scored after 90 min of incubation at room temperature.

<sup>d</sup> HAs similar to those of rabbit erythrocytes after bacterial incubation for 3 h, except that the addition of EDTA (or EGTA) at 5 mM gave much higher HA titers.

<sup>e</sup> ND, Not done.

production by the 24-well plate method. When the strains were grown on CFA agar (supplemented with 3% NaCl) at 37°C, the production of at least two distinct types of HAs was confirmed. One was detected by using either human or guinea pig erythrocytes, was resistant to L-fucose and (moderately) sensitive to D-mannose, and required Ca<sup>2+</sup> for HA activity. This type of HA was found in greater quantities after ca. 3 h than after ca. 20 h of incubation (Table 1). The HA reaction reached a maximal level after ca. 30 min of

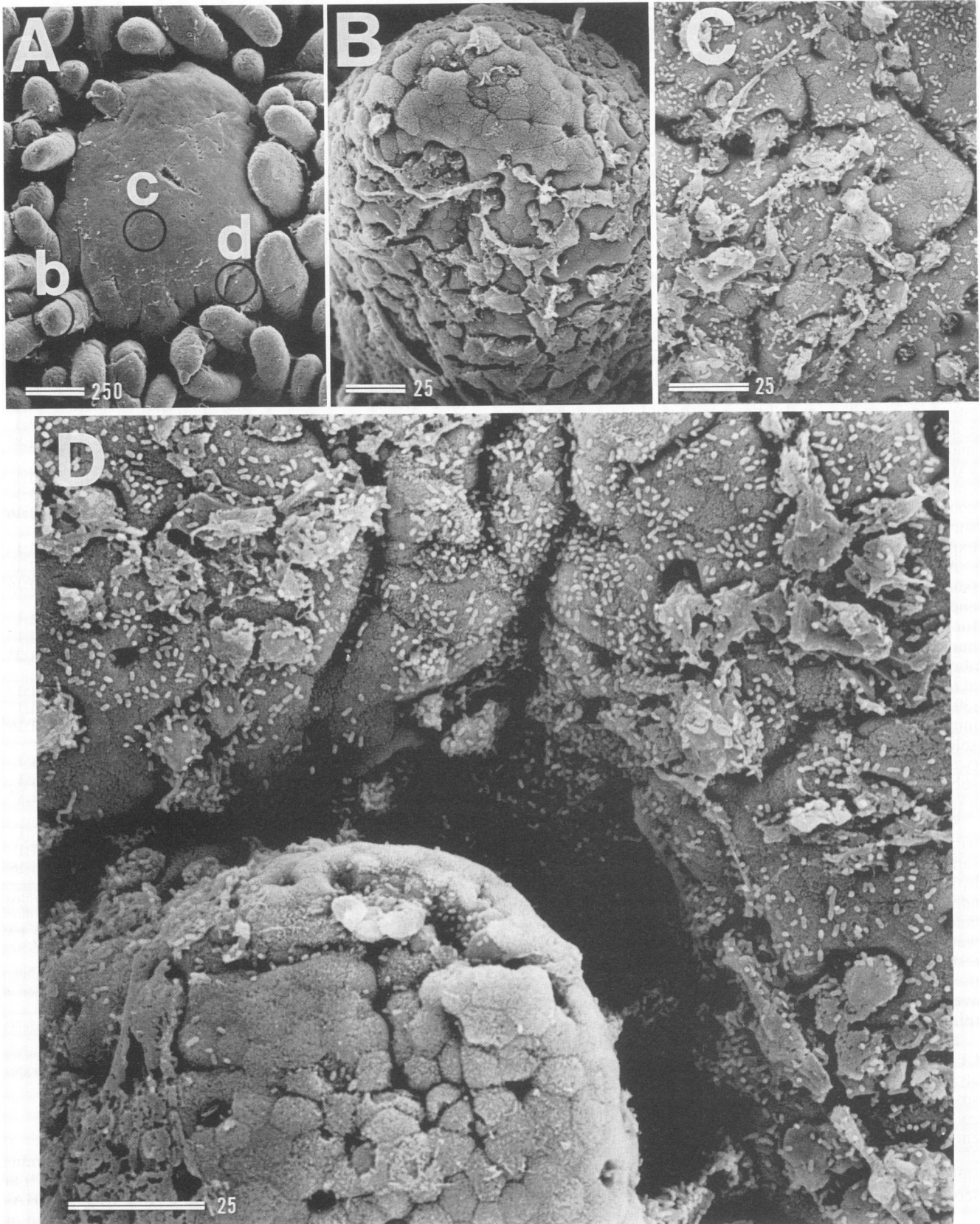


FIG. 1. Adherence of *V. parahaemolyticus* 100A, grown for ca. 3 h (A to E) or 20 h (F) at 37°C, to Formalin-fixed human ileal mucosa (scanning electron micrograph). (A) Lymphoid follicle epithelium surrounded by villi; (B) villus (with mucus), a higher magnification of (e.g.) circle b in panel A; (C) lymphoid follicle epithelium (with mucus), a higher magnification of (e.g.) circle c in panel A; (D) lymphoid follicle epithelium (with mucus) and villus (with mucus) located adjacent to each other, a higher magnification of (e.g.) circle d in panel A; (E and F) lymphoid follicle epithelium (with mucus). Bars, 5, 25, or 250  $\mu\text{m}$ .

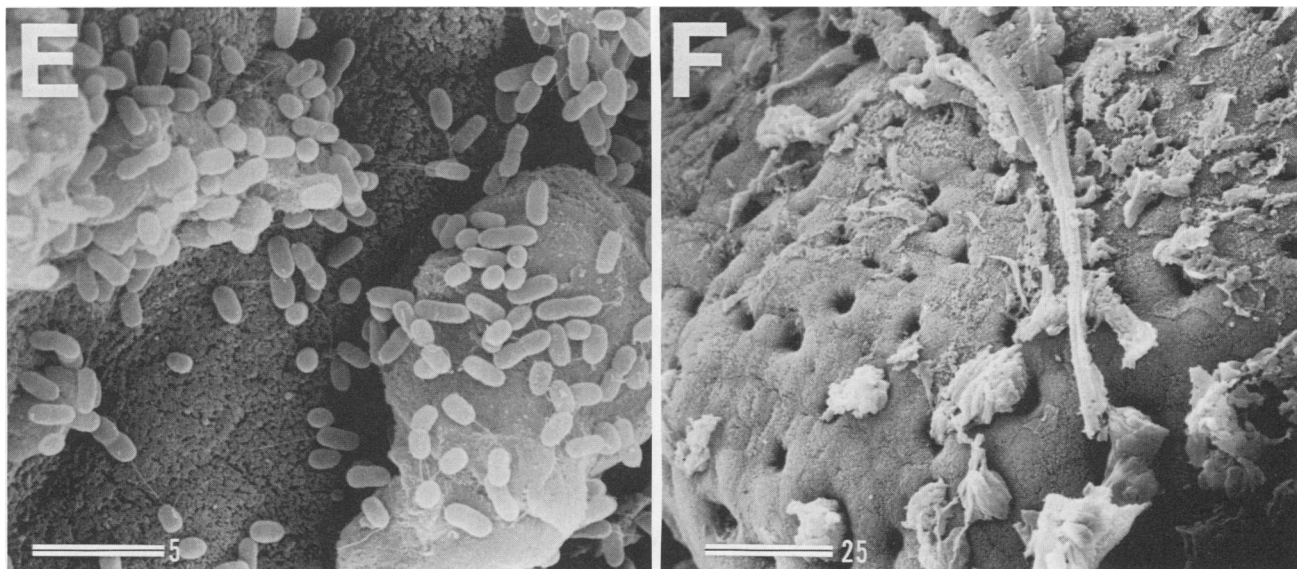


FIG. 1—Continued.

incubation at room temperature; the microtiter plate method gave negative results.

The second type of HA was detected by using rabbit erythrocytes and was resistant to both L-fucose and D-mannose and also (relatively) resistant to treatment with  $\text{Ca}^{2+}$ -chelating agents (Table 1). It was produced to a great extent even after ca. 20 h of incubation (Table 1), and the HA reaction reached a maximal level after 60 to 90 min of incubation at room temperature.

The first type of HA was produced by the strains at various levels, while the second type was produced by all the strains tested at a very high level (Table 1). Neither bovine or sheep erythrocytes were agglutinated by strains under the test conditions.

**Adherence to Formalin-fixed human small-intestinal mucosa.** Formalin-fixed human ileal mucosa with villi and lymphoid follicle epithelium at the mucosal surface (Fig. 1A) was exposed to *V. parahaemolyticus* 100A cells grown on CFA agar (supplemented with 3% NaCl) for ca. 3 or 20 h at 37°C. The 100A cells grown for ca. 3 h adhered to some extent to the epithelial surface of villi (Fig. 1B), but more strikingly they adhered to the epithelial surface of lymphoid follicle epithelium (Fig. 1C); see Fig. 1D for more precise comparison. Adherence to mucus covering the epithelium was at an extremely low level (Fig. 1C and D) except for occasional cases (Fig. 1E). In contrast to the 100A cells grown beforehand for ca. 3 h, the 100A cells grown for ca. 20 h possessed a severely reduced ability to adhere to the mucosal surface including lymphoid follicle epithelium (Fig. 1F).

Similar experiments (using Formalin-fixed human ileal mucosa) were carried out with 10 *V. parahaemolyticus* strains (Table 1), and each adherence index was determined (Table 2); in those adherence experiments, incubation of intestine specimens with bacterial cells was conducted for 10 min at 28°C, and similar results were obtained when incubation was conducted for 30 min at 28°C (data not included). In each case, bacterial cells adhered more extensively to the epithelial cell surface of lymphoid follicles than to the epithelial cell surface of villi. Moreover, ca. 3-h-grown bacterial cells possessed a greater adherence ability than ca. 20-h-grown bacterial cells of the same strains. When bacterial strains grown for ca. 3 h at 37°C were examined, a

correlation (Fig. 2) was obtained between the adherence index and cellular HA levels which were determined with human or guinea pig erythrocytes (Table 1) (though the HA levels may be very low to discuss such a correlation).

TABLE 2. Adherence of *V. parahaemolyticus* strains to the epithelial surface of Formalin-fixed human and rabbit ileal mucosa

Strain	Intestine	Adherence index <sup>a</sup> of indicated epithelium for <i>V. parahaemolyticus</i> strains grown at 37°C for:			
		3 h		20 h	
		Ileal villi	Ileal lymphoid follicles	Ileal villi	Ileal lymphoid follicles
1013	Human	2.5 ± 5.0 0.9 ± 1.7	61.5 ± 59.5 35.8 ± 34.8	1.2 ± 2.0	ND <sup>b</sup>
1014	Human	3.3 ± 4.2 3.8 ± 4.9	58.0 ± 50.4 86.0 ± 79.0	0.2 ± 0.6	4.1 ± 4.1
1015	Human	4.4 ± 6.2	60.1 ± 71.7	0.8 ± 1.5	ND
100A	Human	0.8 ± 1.9 1.9 ± 4.2	73.5 ± 55.4 43.5 ± 45.3	0.3 ± 0.6	0.9 ± 1.2
100B	Human	3.6 ± 3.9 3.2 ± 6.4	73.0 ± 65.8 107.4 ± 62.0	0.1 ± 0.4	0.6 ± 0.9
	Rabbit	3.1 ± 3.2	74.6 ± 52.9	ND	18.8 ± 7.5
1001	Human	0.5 ± 1.0	3.1 ± 5.0	0.1 ± 0.4	0.3 ± 0.9
1006	Human	2.6 ± 6.2	10.8 ± 10.3	0.3 ± 0.8	0.2 ± 0.8
1002	Human	0.3 ± 0.8 0.2 ± 0.8	2.0 ± 2.9 1.6 ± 2.0	0.0 ± 0.0	0.4 ± 1.1
1007	Human	8.7 ± 24.3	15.1 ± 12.7	0.1 ± 0.3	1.1 ± 1.5
1016	Human	1.0 ± 1.9 3.3 ± 5.6	4.8 ± 4.9 4.3 ± 3.7	0.0 ± 0.2	0.0 ± 0.0

<sup>a</sup> Means ± standard deviations for 30 determinations (in the case of ileal villi) or for 60 determinations (in the case of ileal lymphoid follicles). When 3-h-grown bacteria (strains 1013, 1014, 100A, 100B, 1002, and 1016) were tested, two human specimens obtained from different individuals were used and the adherence index for each specimen was determined.

<sup>b</sup> ND, Not done.

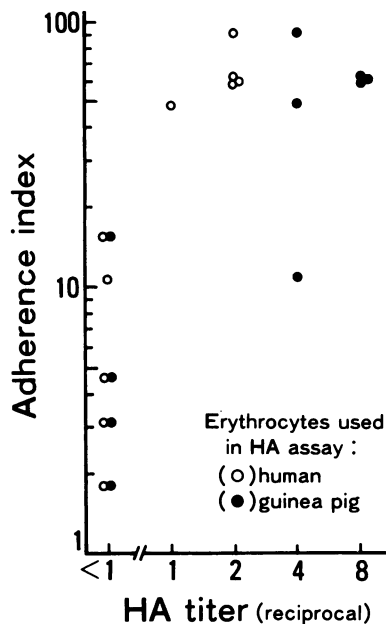


FIG. 2. Relationship between the adherence index for the epithelial cell surface of Formalin-fixed human ileal lymphoid follicles and the *V. parahaemolyticus* cellular HA levels determined with human or guinea pig erythrocytes. Data were obtained with 10 *V. parahaemolyticus* strains (listed in Table 1) which were grown on CFA agar (supplemented with 3% NaCl) for 3 h at 37°C.

Adherence of *V. parahaemolyticus* (strain 100B) to the epithelial cell surface of Formalin-fixed human ileal lymphoid follicles was markedly inhibited by the addition of D-mannose (1% [wt/vol]) or EDTA (5 mM), but not by the addition of L-fucose (Table 3). As mentioned above, *V. parahaemolyticus* occasionally adhered to mucus coating. When mucus adherence of *V. parahaemolyticus* (strain 100B) was tested, no significant inhibitory effect of D-mannose (1% [wt/vol]) was observed.

In adherence experiments, Formalin-fixed human jejunal mucosa was also employed. Adherence levels of *V. parahaemolyticus* strains to the epithelial (absorptive cell) surface of human jejunal villi was similar to those to human ileal villi shown in Table 2 (data not included).

**Adherence to M cells in Formalin-fixed human ileal mucosa.** M cells (located in the lymphoid follicle epithelium of human

TABLE 3. Effect of sugars and EDTA on adherence of *V. parahaemolyticus*<sup>a</sup> to the epithelial cell surface of Formalin-fixed human ileal lymphoid follicles

Addition to assay	Concn	Adherence to epithelial cell type:	
		Cells with microvilli <sup>b</sup>	M cells <sup>c</sup> (n)
None		25.0 ± 18.7	27.3 ± 17.1 (25)
D-Mannose	0.5% (wt/vol)	16.4 ± 25.7	50.9 ± 28.4 (24)
	1.0% (wt/vol)	3.1 ± 4.1	23.2 ± 20.3 (13)
L-Fucose	0.5% (wt/vol)	31.9 ± 28.6	38.3 ± 14.9 (28)
	1.0% (wt/vol)	42.0 ± 47.2	33.9 ± 12.2 (13)
EDTA	5 mM	1.5 ± 1.8	ND <sup>d</sup>

<sup>a</sup> *V. parahaemolyticus* 100B grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C.

<sup>b</sup> Major constituent cells. Adherence index is given (means ± standard deviations for 60 determinations).

<sup>c</sup> Bacterial cell number adherent to the luminal cell surface on an M cell (means ± standard deviations).

<sup>d</sup> ND, Not done.

small intestines) are morphologically characterized by microfolds at the luminal cell surface (22). When human ileal lymphoid follicle epithelium possessing M cells was tested in the present adherence model system, it was found that *V. parahaemolyticus* adhered much more extensively to the surface microfolds of M cells than to the microvilli of the major constituent epithelial cells (Fig. 3). Adherent *V. parahaemolyticus* possessed polar monotrichous flagella (Fig. 3C and D). Such M-cell adherence with *V. parahaemolyticus* (Fig. 3) was enhanced by D-mannose at a concentration of 0.5% (wt/vol) (but not at a concentration of 1%) (Table 3).

**Adherence to Formalin-fixed rabbit ileal lymphoid follicle epithelium.** Figure 4A shows lymphoid follicles, surrounded by numerous villi, in rabbit small intestines. Lymphoid follicle epithelia (indicated with arrows in Fig. 4A to C) are surrounded by tightly folded villuslike structures (Fig. 4B and C). Formalin-fixed specimens of such rabbit ileal mucosa (Fig. 4A) were exposed to *V. parahaemolyticus* 100B cells grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C, and adherence was analyzed by scanning electron microscopy. Again, the best adherence targets for *V. parahaemolyticus* were lymphoid follicle epithelia (Fig. 4D). Adherence to the tightly folded villuslike structures or villi was observed at lower levels. The adherence index determined with *V. parahaemolyticus* 100B (grown for ca. 3 or 20 h at 37°C) is shown in Table 2; ca. 3-h-grown cells manifested much higher adherence levels than did ca. 20-h-grown cells.

**Production of pili.** *V. parahaemolyticus* 1014, which manifested high levels of HA (detected with human or guinea pig erythrocytes) (Table 1) and adherence (Table 2), produced pili when grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h as well as for ca. 20 h at 37°C (Fig. 5). The population of piliated cells was determined by transmission electron microscopy. Of 401 bacterial cells grown for ca. 3 h, 108 (26.9%) were observed to be positive for piliation, while among 215 bacterial cells grown for ca. 20 h, 157 (73.0%) were observed to be positive for piliation, indicating that piliation was more obvious at ca. 20 h of incubation than at ca. 3 h of incubation.

With respect to piliation, results very similar to those of strain 1014 (mentioned above) were obtained with *V. parahaemolyticus* 1013, which manifested high levels of HA (detected with human or guinea pig erythrocytes) (Table 1) and adherence (Table 2) (data not included).

**Motility.** *V. parahaemolyticus* strains (listed in Table 1 or 2) grown on CFA agar (supplemented with 3% NaCl) at 37°C and suspended in KRT for HA or adherence assay were also tested for motility under inverted phase-contrast microscopy. It was found that ca. 3-h-grown bacterial cells were all strikingly motile in contrast to ca. 20-h-grown bacterial cells of the same strains, which showed very weak motility. *V. parahaemolyticus* cells grown for ca. 3 h possessed polar monotrichous flagella (Fig. 3 or Fig. 5).

## DISCUSSION

We have developed an in vitro adherence model system using Formalin-fixed human small-intestinal mucosa (30–33). This model system is advantageous in that one can easily (even though preliminarily) search for bacterial pathogen adherence sites in human small intestines such as mucus coating, villus absorptive cells, or morphologically different epithelial cells (including M cells; 22, 23) over lymphoid follicles (Fig. 6A). This model system is based on earlier observations (12) that *V. cholerae* O1 can adhere to Forma-

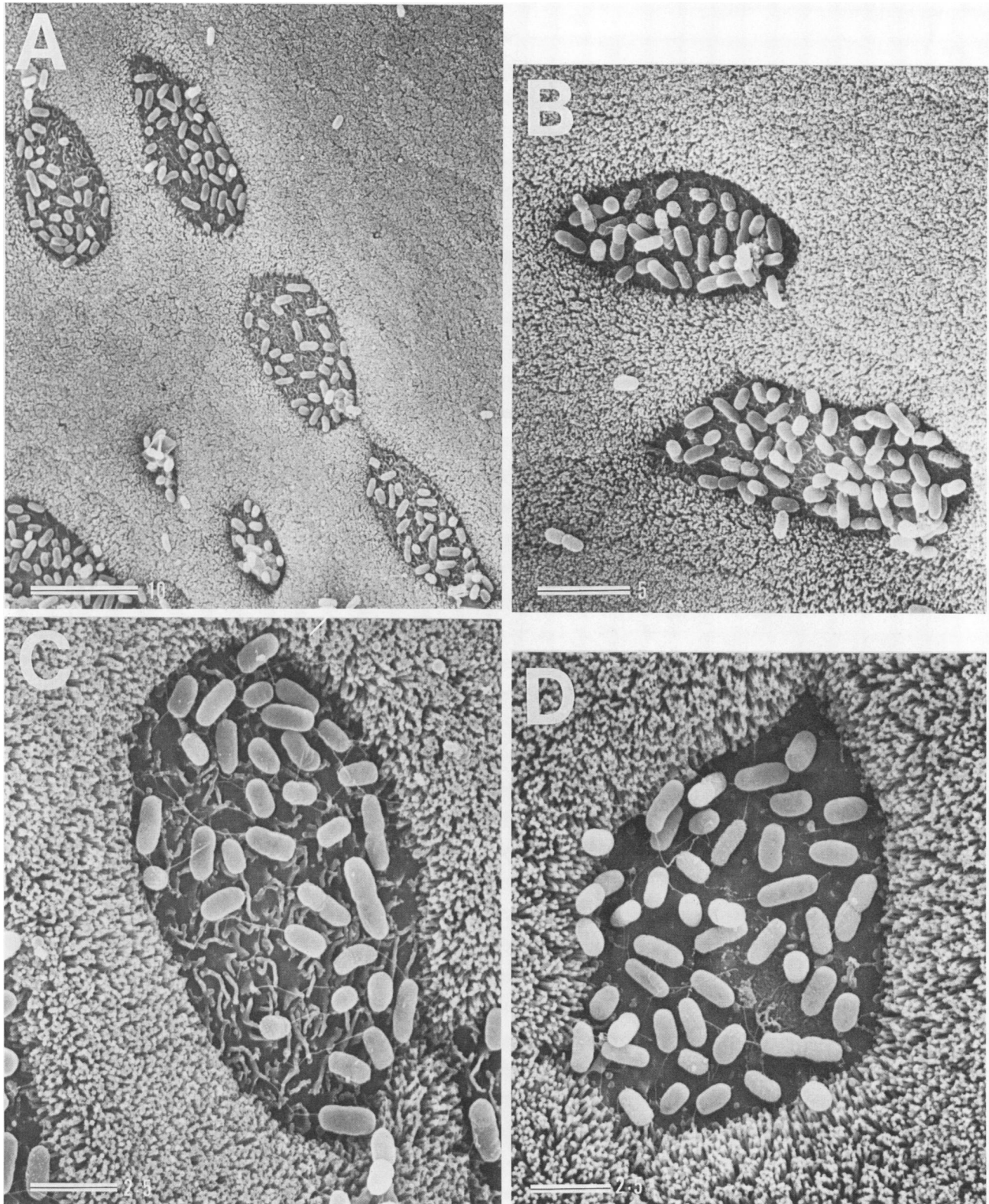


FIG. 3. Adherence of *V. parahaemolyticus* to Formalin-fixed human ileal lymphoid follicle epithelium possessing M cells (scanning electron microscopic analysis). (A to C) M cells with a typical morphology of microfolds which were preferentially adhered with *V. parahaemolyticus*; (D) an epithelial cell with rare microfolds to which *V. parahaemolyticus* adhered. The strain employed was *V. parahaemolyticus* 100B grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C. Bars, 2.5, 5, or 10  $\mu$ m.

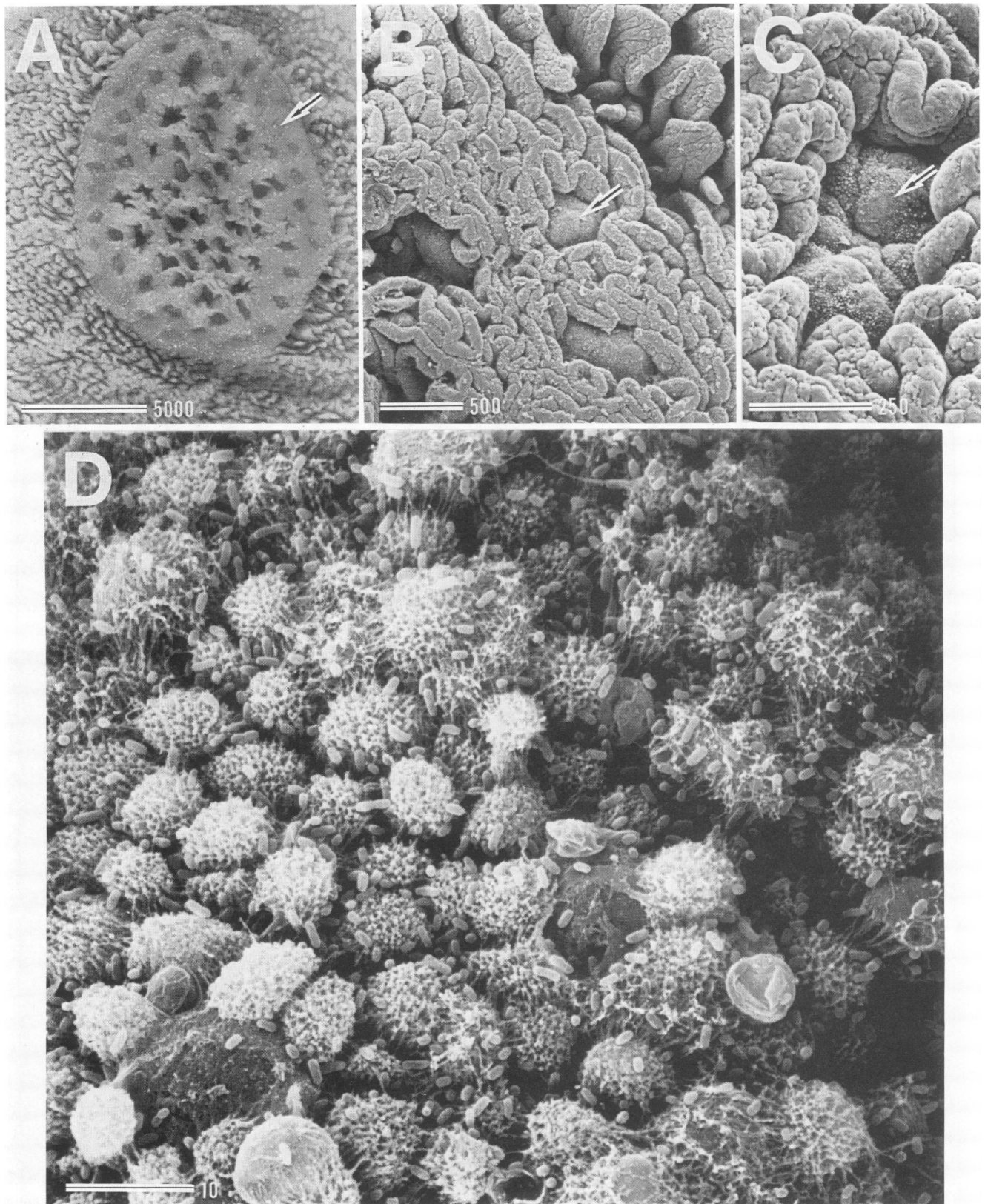


FIG. 4. Rabbit ileal lymphoid follicles (A to C) and lymphoid follicle epithelia (D) to which *V. parahaemolyticus* 100B, grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C, is adhering. (A) Micrograph; (B to D) scanning electron micrograph. Arrows in panels A to C indicate lymphoid follicle epithelia. (D) Lymphoid follicle epithelia (as shown with arrows in B or C) with strain 100B cells adhering. Bars, 10, 250, 500 or 5,000 μm.

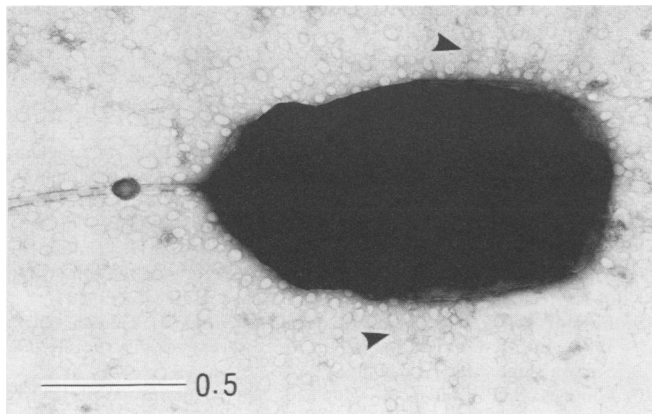


FIG. 5. Transmission electron micrograph of piliated *V. parahaemolyticus* 1014, which was grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C. Arrowheads indicate pili. Bar, 0.5  $\mu$ m.

lin-fixed brush borders of villus absorptive cells to a similar extent as to freshly isolated brush borders, supporting the idea that bacterial pathogens, including *V. cholerae* O1, recognize sugar residues of receptors with their adhesins (1).

The present study, together with previous data (30–32), clearly demonstrates adherence sites which are unique to or common between *V. parahaemolyticus* and *V. cholerae* O1 (or non-O1). Mucus coating, a primary adherence target for *V. cholerae* O1 (or non-O1) in human infection (31, 32), was not adhered much with *V. parahaemolyticus* under the test

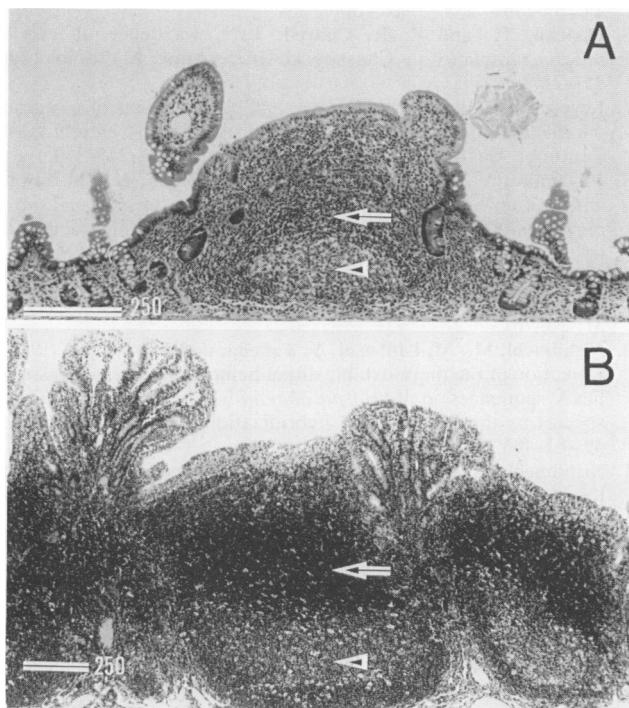


FIG. 6. Histology of human (A) and rabbit (B) ileal lymphoid follicles. Thin ( $\sim 3\text{-}\mu\text{m}$ ) sections of ileal specimens as shown in Fig. 1A (for human lymphoid follicles) and Fig. 4A (for rabbit lymphoid follicles) were prepared and stained with hematoxylin and eosin. Arrows and arrowheads, respectively, indicate lymphocytes and germinal center. Bars, 250  $\mu$ m.

TABLE 4. Summary of adherence sites in human small intestines for *Vibrio* species

Bacterial species	Adherence levels <sup>a</sup> at:		
	Mucus coat	Villus epithelium	Lymphoid follicle epithelium
<i>V. parahaemolyticus</i> <sup>b</sup>	- to +	+	++
<i>V. cholerae</i> O1 <sup>c</sup>	>+++	++	+++
<i>V. cholerae</i> non-O1 <sup>d</sup>	>+++	++	+++

<sup>a</sup> Adherence levels (adherence index): >+++ , >300; +++ ,  $\sim 200$  to 300; ++ ,  $\sim 100$ ; + ,  $\sim 10$ ; - , <0.1.

<sup>b</sup> Data of (e.g.) strain 100B grown on CFA agar (supplemented with 3% NaCl) for ca. 3 h at 37°C and manifesting an HA titer of 1:2 (with human erythrocytes) or 1:4 (with guinea pig erythrocytes) (Tables 1 and 2).

<sup>c</sup> Data of (e.g.) strain C13 (classical biotype; Inaba serotype) grown on CFA agar for ca. 3 h at 37°C and manifesting an HA titer of 1:64 (with human erythrocytes) (30, 31).

<sup>d</sup> Data of (e.g.) strain TVN-324 grown on CFA agar for ca. 3 h at 37°C and manifesting an HA titer of 1:64 (with human erythrocytes) (32).

conditions (Table 4). However, like *V. cholerae* O1 (or non-O1), among small intestinal epithelia tested, lymphoid follicle epithelium provided the best adherence sites for *V. parahaemolyticus* (Table 4). Moreover, M cells were a better adherence target for *V. parahaemolyticus* than were other epithelial cells of the lymphoid follicles (Fig. 3), similar to the case of *V. cholerae* O1 (T. Yamamoto and T. Yokota, J. Infect. Dis., in press). Those data, together with the fact that M-cell microfolds lacked receptors for CFA type I (CFA/I) pili or CFA/II pili of enterotoxigenic *Escherichia coli* (T. Yamamoto, K. Fujita, and T. Yokota, submitted for publication), strongly indicate that chemical structures (e.g., receptors) are different between M-cell microfolds and the microvilli of major constituent epithelial cells. The role of M-cell adherence in pathogenesis must be further precisely characterized, since *V. parahaemolyticus* invades human intestinal tissue (3, 11) and rabbit tissues (4, 5).

As with *V. cholerae* O1 (30, 31) or *V. cholerae* non-O1 (32), *V. parahaemolyticus* more extensively manifested HA activities (Table 1), adherence ability (Fig. 1 and Table 2), and motility after ca. 3 h than after ca. 20 h of incubation at 37°C. It seemed very likely that motility contributed to the high level of adherence manifested by ca. 3-h-grown *V. parahaemolyticus* to some extent; motility alone did not cause adherence (e.g., 3-h-grown strain 1002 [Tables 1 and 2] possessed high motility and manifested very low levels of HA [detected with human or guinea pig erythrocytes] and adherence).

Of the two different types of HAs (Table 1) produced by *V. parahaemolyticus* under the test conditions, only HAs detected with human or guinea pig erythrocytes were correlated with adherence to human ileal lymphoid follicle epithelium (Fig. 2). In accordance with the sugar inhibition pattern observed in the HA reaction with human or guinea pig erythrocytes (Table 1), D-mannose at a concentration of 1% had a marked (but not complete) inhibitory effect on *V. parahaemolyticus* adherence to human ileal lymphoid follicle epithelium (major constituent epithelial cells with microvilli) (Table 3). In contrast, effects of D-mannose on *V. parahaemolyticus* adherence to M cells were stimulatory at a concentration of 0.5% (but not at a concentration of 1%) (Table 3). This contradiction may be due to the different chemical structures (e.g., receptors) of the two adherence targets (microfolds and microvilli). Enhancement of adherence by D-mannose has been noted with some strains of *V. cholerae* O1 (30, 31). HA activities detected with rabbit erythrocytes (Table 1) may also contribute to *V. parahaemolyticus*



*molyticus* adherence to human small-intestinal epithelium to some extent. An example would be the data of strain 1007, which showed relatively high adherence levels (Table 2) and manifested very low HA levels with human or guinea pig erythrocytes, but very high HA levels with rabbit erythrocytes (Table 1). *V. parahaemolyticus* also adhered to the epithelia of rabbit lymphoid follicles (Fig. 6B), as shown in Fig. 4D. Preliminary data indicated that such adherence levels (of strain 100B grown for ca. 3 h at 37°C) were not much affected by the addition of sugars or EDTA under the test conditions; the adherence index (means  $\pm$  standard deviations for 30 observations) was  $93.6 \pm 62.6$  (control),  $99.9 \pm 45.3$  (with 0.5% [wt/vol] D-mannose),  $65.4 \pm 32.1$  (with 1% [wt/vol] D-mannose),  $79.0 \pm 42.8$  (with 0.5% [wt/vol] L-fucose),  $57.6 \pm 30.4$  (with 1% [wt/vol] L-fucose), and  $30.0 \pm 18.6$  (with 5 mM EDTA). The possible role of the two types of HAs in adherence in a rabbit model is under further investigation.

We confirmed the piliation of *V. parahaemolyticus* (strains 1014 and 1013) grown on CFA agar (supplemented with 3% NaCl) at 37°C (Fig. 5 and text). Such piliation was observed more extensively at ca. 20 h of incubation, when no detectable HA (with human or guinea pig erythrocytes) was produced (Table 1) and very low levels of adherence were observed (Table 2), than at ca. 3 h of incubation, when levels of HA (with human or guinea pig erythrocytes) as well as adherence (to ileal lymphoid follicle epithelium) were markedly high (Tables 1 and 2).

When CFA/I- or CFA/II-possessing enterotoxigenic *E. coli* was tested under the present adherence model system, bacterial cell-bound CFA/I or CFA/II pili, which attached to microvilli at the pilus free end (pilus tip side), were clearly seen in a scanning electron microscope (33). In the case of adherent *V. parahaemolyticus*, however, bacterial cell-bound pili which attached to microfolds or microvilli at the pilus free end (pilus tip side) were not confirmed in a scanning electron microscope under the test conditions (e.g., Fig. 3C and D). Therefore, as far as the present in vitro adherence model system is concerned, high levels of motility (due to terminal polar flagella) and HA activity (detected with human or guinea pig erythrocytes) seemed to be more important for *V. parahaemolyticus* adherence than pili produced under the present test conditions.

The HA activities and adherence abilities of *V. parahaemolyticus* Kanagawa phenomenon-negative strains isolated from the environment are under investigation.

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