INFECTION AND IMMUNITY, Sept. 1987, p. 2093–2102
0019-9567/87/092093-10$02.00/0

Vol. 55, No. 9

In Vivo Adherence and Colonization of *Vibrio cholerae* Strains That Differ in Hemagglutinating Activity and Motility

JACOB S. TEPPEMA,* PIET A. M. GUINÉE, AWAD A. IBRAHIM,† MARCEL PÂQUES, AND E. JOOST RUITENBERG

National Institute of Public Health and Environmental Hygiene, 3720 BA Bilthoven, The Netherlands

Received 24 November 1986/Accepted 8 June 1987

A scanning electron microscopic study was carried out to compare the in vivo pathogenicity of two strains of *Vibrio cholerae* in an adult rabbit ligated-gut test model, *V. cholerae* C5 (serotype Ogawa, biotype El Tor), a motile strain possessing hemagglutinating activity in vitro, and C21 (serotype Ogawa, classical biotype), a nonmotile strain possessing no hemagglutinating activity, were tested. Tissue samples from small intestinal loops were examined 3, 6, 9, and 12 h postinoculation. Contradictory to most published data, neither hemagglutinating activity nor motility appeared to be essential prerequisites for the pathogenesis of cholera in the experimental animal model used: nonmotile hemagglutinin-negative strain C21 adhered to and colonized the small intestine at least to the same extent as did motile hemagglutinin-positive strain C5. Maximum colonization was seen at 9 h postinoculation for both strains. C5 and C21 vibrios caused comparable damage to the villi of the small intestine. The villous epithelium showed only mild changes during the first 9 h postinoculation. However, after 12 h the epithelium was seriously damaged concomitant with a decrease in the number of vibrios. Many villi showed partial or total denudation, owing to repelled epithelium, leaving a bare basal lamina with only some to moderate numbers of vibrios attached. Since similar changes were induced by pure cholera enterotoxin, these changes were likely the result of excessive fluid accumulation. From this study it is concluded that, at least in the animal model used, factors other than hemagglutinating activity and motility may also play a role in the association of *V. cholerae* with the small intestinal surface.

Cholera is an infectious diarrheal disease caused by *Vibrio cholerae*, a noninvasive bacterium that adheres to and multiplies on (colonizes) the small intestinal epithelium (16, 32, 37, 39). Clinical symptoms are ascribed to the production and release of a toxin that, by internalization into the gut epithelial cells (24), activates adenylate cyclase, thus accelerating cyclic AMP pathways and leading to a net secretion of electrolytes and water into the gut lumen (13, 25, 41).

Because cholera is an endemic disease in many parts of the world, the search for new and efficient vaccines is proceeding with strength (30). Within this scope two of the main objectives are (i) the development of animal models suitable for studying the pathogenicity of *V. cholerae* and for measuring protection against experimental cholera following immunization and (ii) characterization of different *V. cholerae* strains with respect to their enterotoxigenicity and hence their virulence, combining in vitro and in vivo methods.

One of the animal models explored as a method for studying the events leading to the disease was the adult rabbit ligated-gut test (ARLGT) first described by De and Chatterjee (8). In the course of our investigations, several *V. cholerae* strains were tested in a standardized model of this test as well as in some modifications (21) of the removable intestinal tie adult rabbit diarrhea (RITARD) model of Spira et al. (38). Among the *V. cholerae* strains tested were those having mannose-resistant hemagglutinating activity (HA^+) as well as those lacking this activity (HA^-) and motile as well as nonmotile strains. In vitro hemagglutinating activity has been suggested to be correlated with adherence capacity (16, 27, 28) and perhaps also with the presence of fimbriae, as is the case with some enteropathogenic *Escherichia coli* and other bacteria (4, 33). Furthermore, it has been suggested that association with mucosal surfaces is dependent on the capacity for motility (19, 28, 42).

In the present study we report on the adherence of two strains of *V. cholerae*, C5 (HA^+, motile) and C21 (HA^-, nonmotile), in a modified ARLGT, as observed by scanning electron microscopy (SEM). We show that strain C21 vibrios adhere to and colonize the small intestinal epithelium at least to the same extent as do strain C5 vibrios and cause comparable damage to the gut mucosal epithelium, despite the absence of hemagglutinating activity and motility in strain C21 vibrios.

MATERIALS AND METHODS

Animals. Outbred male New Zealand White rabbits (ENKI or Broekman, Someren, The Netherlands) weighing 2 to 2.5 kg were used. The animals were kept individually in wire cages at 21°C under conventional conditions with a light-dark cycle of 11 and 13 h, respectively, and received 80 g of food pellets (Hope Farms, Woerden, The Netherlands) daily and tap water ad libitum. The animals were observed for 2 weeks, and only healthy-looking animals were used for the experiments. The animals were starved for 24 to 48 h prior to the start of the experiments.

Bacteria. *V. cholerae* C5 (serotype Ogawa, biotype El Tor) and C21 (serotype Ogawa, classical biotype) were used. Both strains were field isolates belonging to the culture collection of the National Institute of Public Health and Environmental Hygiene. Strain C5 was isolated in 1957 during an outbreak of cholera in Indonesia, and strain C21 was isolated in Dacca in 1963. The strains had been kept since then in the lyophilized state until used for the work described here and elsewhere (21). Strain C5 was HA^+ and motile, as described earlier (21), and possessed a polar

* Corresponding author.
† Present address: Central Veterinary Research Labs Ammart, Khartoum, Sudan.

flagellum, as confirmed by negative-contrast transmission electron microscopy (J. S. Teppema, unpublished observation). Strain C21 was HA^-, spontaneously nonmotile, and aflagellate. A third *V. cholerae* strain, 35A3 (serotype Inaba, classical biotype), which had weak haemagglutinating activity and was motile (21) and which was used for vaccine production at our institute, was chosen for control purposes. C5, C21, and 35A3 were all enterotoxin-producing strains (21).

Samples of stock cultures stored at −70°C in nutrient broth containing 15 to 20% glycerol were thawed and grown overnight at 37°C in Evans synthetic medium (9). The respective titers for mannose-resistant haemagglutinating activity, as determined with bacteria grown in liquid Evans synthetic medium with aeration (250 strokes per min) at 25°C for 20 h and freshly collected human type O erythrocytes, were 1:128 for C5, 1:8 for 35A3, and <1:4 for C21 (21). For inoculations 2-ml bacterial suspensions containing 10^6 cells per ml in Evans synthetic medium were used.

ARLGT. A modified version of the model developed by De and Chatterjee (8) was used. Details of the anesthesia and surgical procedures are described elsewhere (A. A. Ibrahim, Ph.D. thesis, University of Utrecht, Utrecht, The Netherlands, 1984). Briefly, a primary ligature of the small intestine was made approximately 90 to 100 cm anterior to the appendix, and the selected part of the jejunum-ileum was flushed with sterile phosphate-buffered saline (PBS). Five or six 10- to 12-cm loops with interloop spacings of at least 2 cm were made in the ileal and jejunal parts of the small intestine of each of eight rabbits, starting approximately 10 cm proximal to the ileocecal transition. The loops were injected in arbitrary sequence with the *V. cholerae* C5 or C21 challenge material, taking care that adjacent loops were never inoculated with the same challenge material. However, some loops were injected with 2 ml of PBS to serve as negative controls, and other loops were inoculated with *V. cholerae* 35A3 or with 2 ml of PBS containing 10 μg of purified cholera toxin (generous gift from Wellcome Research Laboratories, Beckenham, England) to serve as positive controls.

At 3, 6, 9, or 12 h postinoculation (p.i.), the animals were killed by injection with an overdose of pentobarbital, the abdominal cavity was opened, and the fluid contents of loops at 12 h p.i. only were removed for measurement of the fluid accumulation (loops at earlier times showed only low to moderate fluid accumulation). Tissue samples from the intestinal loops were collected in duplicate for SEM studies. In all, 43 loops were prepared: 10 from two rabbits at 3 h p.i. (1 PBS, 2 C5, and 2 C21 loops per animal); 6 from one rabbit each at 6 and 9 h p.i. (3 C5 and 3 C21 loops per animal); and 21 from four rabbits at 12 h p.i. (6 C5, 6 C21, 3 PBS, 3 toxin, and 3 35A3 loops).

SEM. Excised ring segments of the intestinal loops were cut open longitudinally under PBS, trimmed to pieces of 1 to 2 cm^2, and pinned flat on paraffin under PBS. The pieces of intestine were washed by gentle agitation in four consecutive changes of PBS to remove debris and any bacteria not firmly bound. Subsequently, PBS was replaced by 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and the samples were fixed at 4°C for 4 h. Alternatively, pieces of intestinal tissue were cut open and pinned flat on paraffin under glutaraldehyde fixative, without being washed, and were fixed in the same way. Thus, both unwashed and washed intestinal preparations were available for examination.

After fixation pieces of intestinal tissue measuring approximately 0.25 to 0.5 cm^2 were cut with razor blades under fixative buffer. These were then washed by immersion without agitation in several changes of the buffer with 0.2 M sucrose added and were stored at 4°C until further preparation.

The samples were immersed twice for short times in fixative buffer prior to postfixation, postfixed in 0.1 M sodium cacodylate-buffered 1% OsO_4 (pH 7.4) at room temperature for 60 min, washed once with distilled water, and dehydrated in a graded series of acetone, ending with 100% acetone for 20 min twice. The samples were transferred to a Polaron E 3000 critical point drying apparatus, dried with CO_2 (three flushes of 2 min each; total duration of CO_2 critical point drying treatment, 2 h), mounted on aluminum stubs, and gold-sputtered in a Polaron E 5000 sputter coating apparatus. The specimens were examined in a Philips 501 B SEM operated at 7.2 to 30 kV.

TABLE 1. Fluid accumulation in ligated intestinal loops of adult rabbits 12 h p.i.

Loops inoculated with:	Fluid accumulation ratio[a]
PBS	—[b]
Strain C5	1.75 ± 0.34
Strain C21	2.23 ± 0.25
Strain 35A3	1.53 ± 0.13
Cholera toxin	2.93 ± 0.23

[a] Volume (milliliters) of fluid present per centimeter of loop. Data represent the mean ± standard deviation in three rabbits.
[b] —, No fluid accumulation present.

RESULTS

At 3 h p.i. no fluid had accumulated in the loops. At 6 and 9 h p.i. the loops inoculated with *V. cholerae* C5, C21, or 35A3 or with purified cholera toxin showed low to moderate fluid accumulation. However, at 12 h p.i. a large amount of fluid had accumulated in these loops (Table 1).

The SEM observations described hereafter represent primarily the interaction between vibrios and the small intestinal epithelial surface and the villous epithelial changes observed. A summary of the results is shown in Table 2.

Loops 3 h p.i. (i) Negative control loops (PBS injected). At 3 h p.i. negative control loops showed normal jejunal or ileal villi with numerous, mostly transverse furrows, possibly a result of villous contraction (Fig. 1a). Higher magnifications revealed individual polygonal epithelial cells (Fig. 1b) and occasional goblet cells. Tips of microvilli of epithelial cells were observed at high magnifications, giving the cell surface a rather granular appearance. Occasionally, groups of microvilli sticked together or microvilli appeared to be more or less obscured by a thin, granular, amorphous layer, possibly the glycocalyx. Sometimes, extrusion of cells at villous tips, known to occur normally in many species (35), was evident. The villous base and crypt orifices could not be observed, the villi being close to each other. Adherent bacteria were virtually absent.

(ii) C5- and C21-infected loops. Villi seemed grossly unchanged in comparison with those from the control loops. However, some villi appeared slightly roughened, with single epithelial cells slightly protruding above the villous surface and microvilli sticking together in groups more clearly. At other sites the granular amorphous layer over the microvilli was more prominent.

TABLE 2. Summary of SEM results

Material used to inoculate loops and time (h) of examination p.i.	SEM result[a]										
		Condition of villous epithelium					*V. cholerae*				
	Gross pathology of intestinal villi	Roughened appearance (irregular microvilli)	Swollen and/or protruding cells, cell blebs	Enhanced cell extrusion	Denudation, bare basal lamina	Loose epithelial cell aggregates	Presence of vibrios	Solitary epithelial adherence	Epithelial colonization	Attachment to bare basal lamina	Attachment to loose cells or cell aggregates
PBS											
3	−	−	−	−	−	−	−	−	−	−	−
12	+	+	±	±	−	+	−	−	−	−	−
C5 (HA^+, motile)											
3	−/+	±	−	−	−	−	+	+	±	−	−
6	−/+	±	±	−	−	−	+(+)	+	+(+)	−	−
9	+	+	±	+	−	±	++(+)	+	++	−	−
12	++(+)	++	+(+)	++	++	++	++(+)	−	+(+)	+(+)	++
C21 (HA^-, nonmotile)											
3	−/+	±	−	−	−	−	+	+	+	−	−
6	−/+	+	±	−	−	−	++	+	++	−	−
9	+	++	±	±	−	±	+++	+	+++	−	−
12	++(+)	+++	++	+(+)	++	++	++(+)	−	+(+)	+	+
35A3[b], 12	+	+(+)	+	±	−	+	++	±	+(+)	−	+
Toxin, 12	+++	+++	+	++	++	++(+)	−	−	−	−	−

[a] Scoring system: −, absent or not observed; ±, rarely present or observed; +, slightly changed or present to a low degree or in low numbers; ++, moderately changed or present to a moderate degree or in moderate numbers; +++, seriously damaged, prominent, or present to a high degree or in high numbers; −/+, score variable from − to +; +(+), score variable from + to ++; ++(+), score variable from ++ to +++.

[b] Strain 35A3 is a weakly hemagglutinating, motile vibrio (see Materials and Methods).

Cholera bacteria were present in very low numbers, scattered solitarily or in very small groups over the surfaces of the villi (Fig. 2 and 3). Definitely more C21 vibrios than C5 vibrios were detected on the villous surfaces of the respective loops. Strain C21 bacteria were characterized by their elongated, slightly curved (comma-shaped) cell bodies (Fig. 3), and strain C5 bacteria were usually characterized, in addition, by their polar flagella (Fig. 2). C5 vibrios more than C21 vibrios seemed to lie preferentially under the lee of villous folds. The initial contact of vibrios with villous epithelium may well occur with the nonflagellar polar side in the case of C5, since a head-on arrangement of C5 vibrios was seen occasionally (Fig. 2a), with the flagellar pole and flagellum extending into the lumen, as also described by Nelson et al. (32). Often, C5 vibrios as well as C21 vibrios slightly indented the brush border surface, i.e., the glycocalyx or tips of microvilli or both, suggesting firm attachment (Fig. 2b and 3b). At these sites microvilli were often

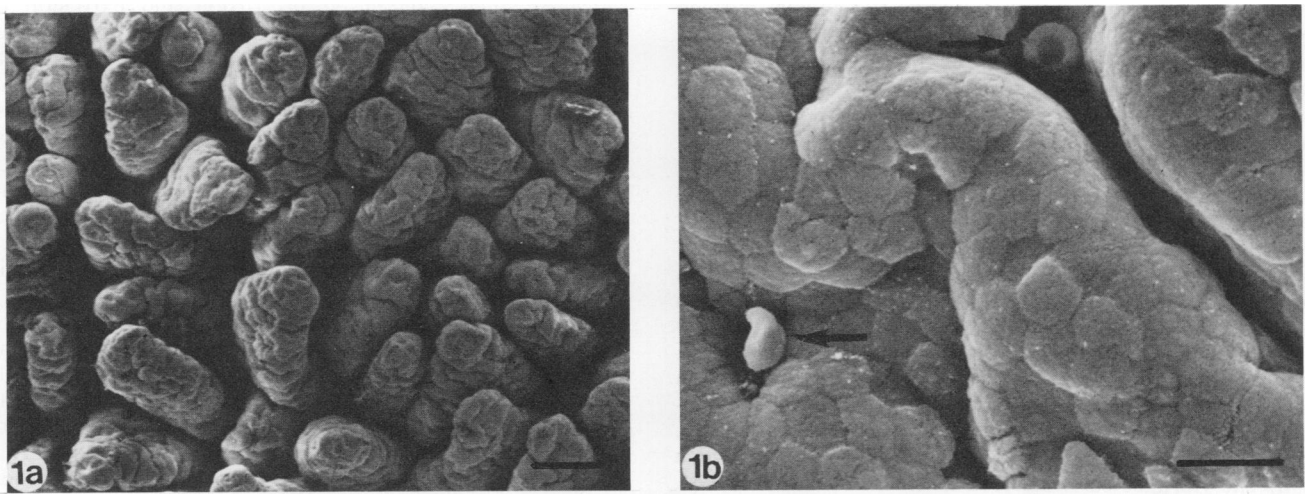

FIG. 1. SEM of normal small intestinal villi from a control (PBS-injected) ligated loop at 3 h p.i. (a) Survey; (b) surface detail showing furrows, the polygonal outline of epithelial cells, and normal or aberrantly shaped erythrocytes (arrows) occasionally present at sheltered sites. Bars, 100 μm (a) and 10 μm (b).

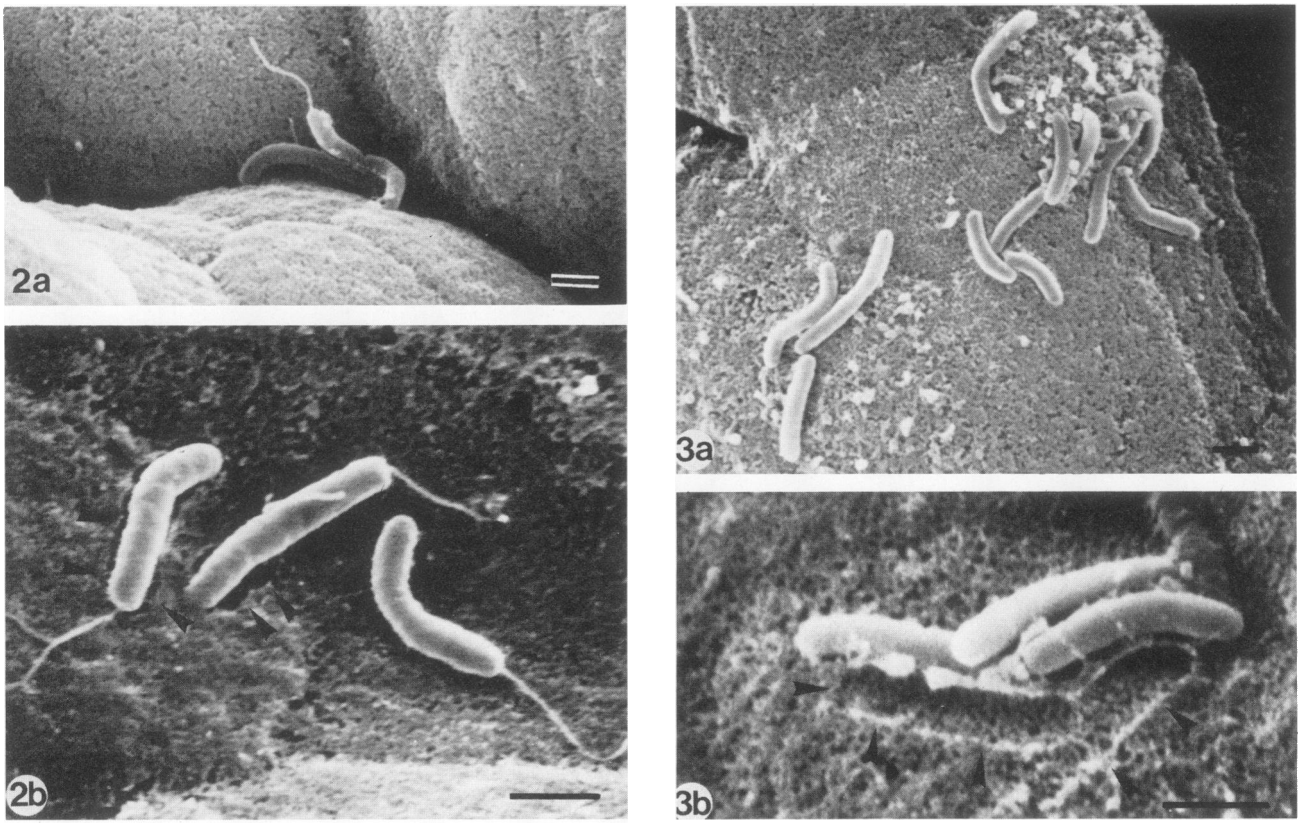

FIG. 2 and 3. Vibrios of strains C5 (Fig. 2) and C21 (Fig. 3) adhering to villous epithelium at 3 h p.i. Note the polar flagellum on C5 (Fig. 2a and b) and indentations (arrowheads) made by bacterial cells in the granular, amorphous layer covering the microvilli (Fig. 2b and 3b). A head-on orientation is suggested for C5 vibrios (Fig. 2a). Bars, 1 μm.

clearly visible, suggesting that the amorphous layer over the microvilli was dissolved by some action of the bacteria.

C5- and C21-infected loops 6 and 9 h p.i. Most villi at these stages appeared almost normal, more or less comparable with those of the C5- or C21-inoculated loops at 3 h p.i. Occasionally, however, enhanced cell extrusion at the villous tips was noted, and single cells were extruded elsewhere from the epithelium. Locally, the villous surface appeared roughened because of epithelial cell swelling and increased microvillous irregularity. Protruding epithelial cells regularly had smooth blebs and showed a partial loss of microvilli. In contrast with the 3-h stage, no glycocalyxlike layer was noted.

In both C5- and C21-inoculated loops, vibrios, including dividing forms, were present over the full villous length, except for the outermost tips. Their numbers varied largely per villus. Although solitary bacteria were noted, colonization was obvious for both strains at 6 h p.i. (Fig. 4 and 5) and considerably more so at 9 h p.i. (Fig. 6 and 7). C21 vibrios outnumbered C5 vibrios, colonies of C21 being more prominent than those of C5. Far more C5 bacteria than C21 bacteria occurred preferentially at sheltered sites, such as villous folds or cavities resulting from cell extrusion. Occasionally, small lumps of aggregated host cells and cell debris, mixed with large numbers of vibrios, were seen in between villi. The vibrios clearly adhered to the microvilli; however, no special orientation or arrangement of the bacteria towards the epithelium was noted. C5 cells as well as C21 cells showed the characteristics described above. At 9 h p.i., however, cells of both strains, but of C5 in particular, had a less smooth surface, and occasional filamentous cholera bacteria were seen.

Loops 12 h p.i. (i) Negative control loops (PBS injected). The villous architecture more or less resembled that of the 3-h control (PBS-injected) loops. However, some villi appeared slightly swollen or were moderately roughened because of slightly increased protrusion and extrusion of epithelial cells. Small lumps of epithelial cells were sometimes present in between villi. Bacteria were virtually absent.

(ii) C5- and C21-infected loops. Dramatic changes had occurred by 12 h p.i. in both C5- and C21-inoculated loops, as compared with the foregoing stages. The morphological damage varied a great deal. Besides villi with the same minor changes as those described above, many partially or completely denuded villi were present, revealing the bare basal lamina. Many stages of the denudation process were noted (Fig. 8). Masses of aggregated epithelial cells were present in between the villi.

Many variations were also noted with regard to the presence of vibrios. Relatively intact villi showed many colonizing vibrios or only a few. Bacteria were mainly present at sheltered sites (Fig. 9a). In general, C21 accumulations were more extensive than those of C5. Few vibrios were seen in areas with many seriously damaged villi. Denuded villi possessed variable numbers of vibrios adhering to the bare basal lamina. Notably more C5 bacteria than C21 bacteria were present at this site (Fig. 9b). These bacteria never showed any sign of colonization, and dividing

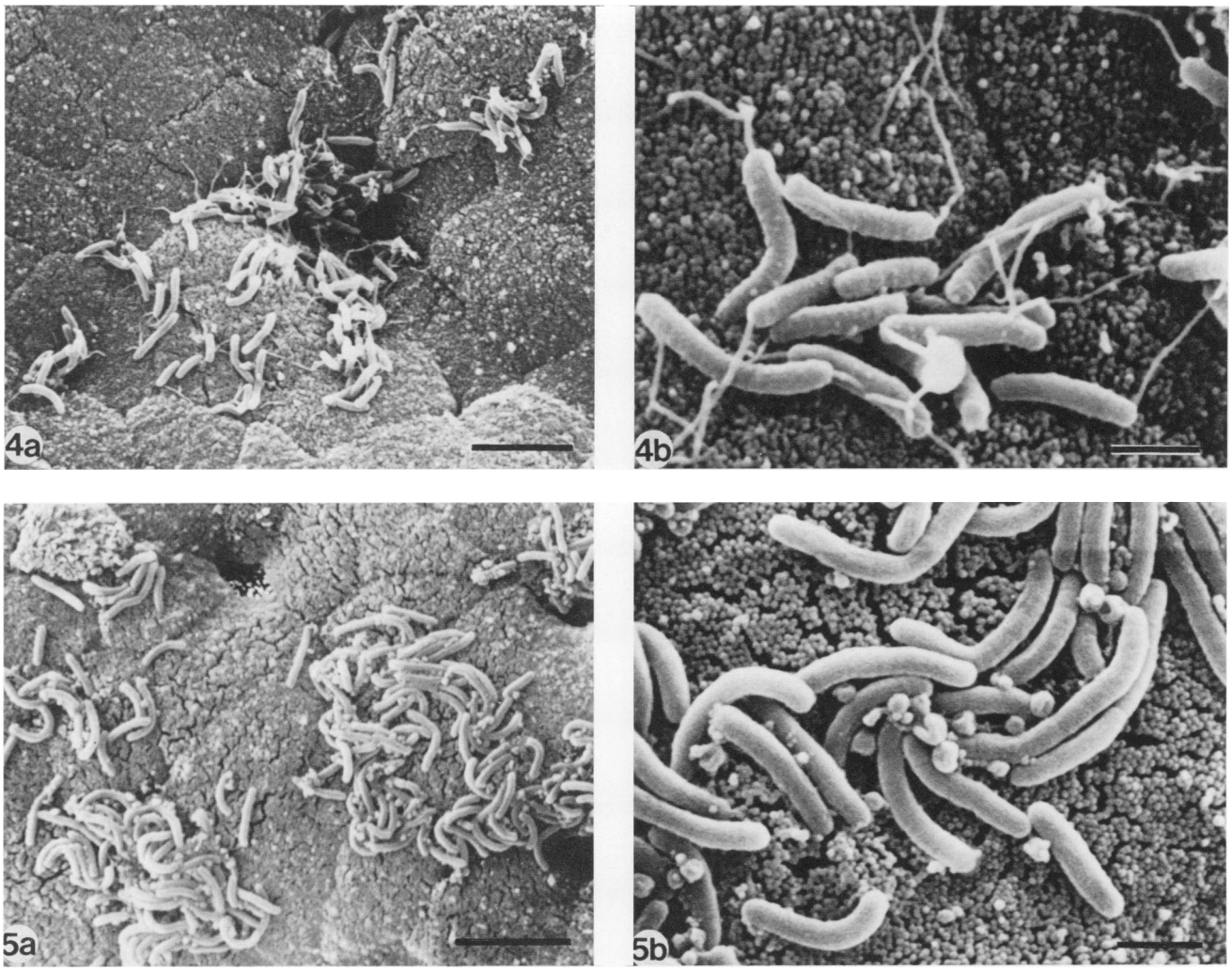

FIG. 4 and 5. Strain C5 (Fig. 4) and strain C21 (Fig. 5) vibrios colonizing villous epithelium at 6 h p.i., colonies of C21 being more prominent than those of C5. Some predilection of C5 vibrios for sheltered sites is apparent in Fig. 4a. Fig. 4b and 5b clearly show the tips of microvilli. Bars, 5 μm (a) and 1 μm (b).

cells were virtually absent. Furthermore, variable numbers of vibrios were found intermingled with the cellular aggregates in between villi (Fig. 9c).

Although difficult to estimate because of the large variations seen, the total numbers of C5 and C21 vibrios seemed reduced substantially as compared with those at the 9-h stage. In addition, and contrary to the 3-, 6-, and 9-h stages, a clear difference was noted between unwashed and washed preparations (see Materials and Methods) with respect to the numbers of vibrios present, far less bacteria being seen in washed preparations. Generally, C5 and C21 vibrios still showed their characteristic structures; however, both were clearly shortened in length in comparison with those at 3 to 9 h p.i., C5 more distinctly than C21, and both were rather smooth.

(iii) Positive control loops. Except for the absence of bacteria, vibriolike forms in particular, the loops injected with purified cholera toxin showed pathological changes at least to the same extent as those injected with C5 or C21 12 h p.i. Many villi appeared greatly roughened, and epithelial cells were extruded individually or in groups; several villi clearly displayed (partial) denudation (Fig. 10a). Loose aggregates of epithelial cells were frequently present, covering part of the villous surfaces. One of the loops was completely hemorrhagic.

In contrast, loops inoculated with the weakly hemagglutinating and motile strain 35A3 vibrios showed only mild morphological changes more or less comparable with those seen in the 6- and 9-h stages of the C5- or C21-infected loops, i.e., a mildly roughened epithelial surface and no signs of villous denudation (Fig. 10b). Moderate numbers of colonizing cholera cells were observed on the villous epithelium (Fig. 10c).

Effect of washing on the mucous blanket and vibrios. Both washed and unwashed preparations of all loops at all time intervals were studied. Unwashed preparations showed variable remnants of a mucous layer over the villi, partially obscuring these, while washed preparations generally showed a fairly clean epithelial surface with only small focal mucous blanket remnants present. The mucous layer consisted of a granular-amorphous or sometimes fibrous layer of variable thickness. At 3 h p.i., no vibrios were found in the mucus. At later stages, i.e., 6 and 9 h p.i., variable numbers of vibriolike cells were observed locally in this layer. Sometimes, however, it was impossible to decide whether the mucous blanket was indeed involved or whether an over-

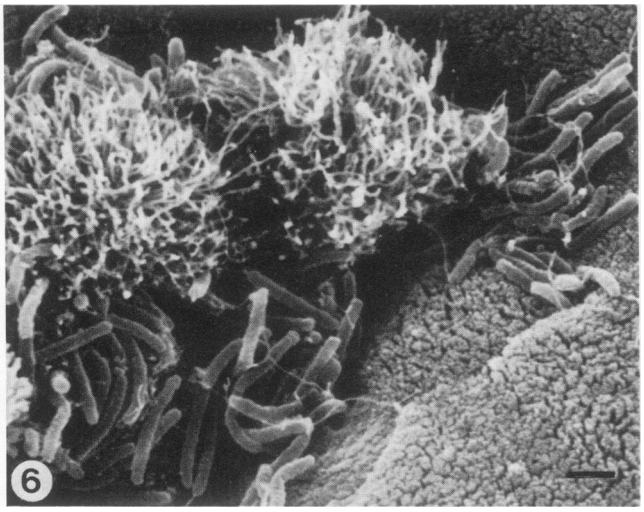

FIG. 6. Colonizing C5 vibrios showing a predilection for a sheltered site near protruding epithelial cells with irregular, hairy microvilli at 9 h p.i. Bar, 1 μm.

whelming colonization of vibrios on the intestinal epithelium, obscuring the latter, was present. Except for the 12-h stage, no differences in the numbers of vibrios were observed between washed and unwashed preparations. The washing procedure appeared unable to remove vibrios from the epithelium, suggesting that vibrios adhered to the surface firmly.

DISCUSSION

The present study shows that HA^-, nonmotile *V. cholerae* C21 is capable of adhering to and multiplying on gut epithelium at least to the same extent as is HA^+, motile *V. cholerae* C5. Strain C5 and, by comparison, the weakly hemagglutinating and motile strain 35A3 behaved more or less as expected. In fact, C21 vibrios were even better able to adhere to the villous epithelium than were C5 vibrios; the latter were present preferentially at sheltered locations, while C21 was less fastidious. As a consequence, colonization of C5 vibrios appeared to be restricted more to these sheltered sites. In all stages studied except 12-h p.i., C21 vibrios outnumbered C5 vibrios.

It appears from the present study that, in the experimental model chosen, neither hemagglutinating activity nor motility, either separately or in combination, is an essential property enabling certain strains of *V. cholerae* to adhere to and colonize the small intestinal epithelium. This conclusion was confirmed by the results of concomitant in vivo experiments on the immunofluorescence of adhesion and bacterial viable counts (A. A. Ibrahim, Ph.D. thesis, University of Utrecht, Utrecht, The Netherlands, 1984). The findings are also fairly compatible with data from a study with a modified RITARD (i.e., open) model, in which *V. cholerae* C21 was moderately virulent, whereas *V. cholerae* C5 and 35A3 were highly virulent and avirulent, respectively (21).

Colonization, i.e., adherence and multiplication, and toxin production are the essential events leading to cholera. Adherence to and colonization of the small intestine by *V. cholerae* have been demonstrated both in vitro and in vivo (16, 32, 37, 39). The results of our SEM study are clearly compatible with those of Nelson et al. (32) and confirm the progressive increase in *V. cholerae* bacteria adhering to the small intestinal villi, with maximum colonization up to about 8 to 12 h p.i., and reduced adherence afterwards, as found in various animal models (12, 20, 32, 39). In the present study, concomitant with a decline in the number of C5 or C21 vibrios, serious damage of the villous epithelium was observed at 12 h p.i.; such damage was not present in earlier SEM studies (20, 32). However, the observations indicate that most of the vibrios detached from the villous epithelium prior to, and not as a result of, the denudation of the villi. The reduction in adherence was not a simple consequence of villous denudation.

Bacterial adherence is mediated by adhesin-receptor interactions (4) and frequently has been correlated with the presence of fimbriae or pili (see references 4 and 33 for reviews) and hemagglutinating activity (33). With regard to *V. cholerae*, too, adherence capacity has been suggested to be correlated with hemagglutinating activity (16, 26–28, 30). There is little evidence, however, that hemagglutinating activity represented by the fimbriae of *V. cholerae* is responsible for adherence, as in the case of various members of the family *Enterobacteriaceae*, e.g., some enteropathogenic *E. coli* (18, 33). Nevertheless, fimbriae on *V. cholerae* strains have been described in the literature (3, 40) and recently by one of us using negative-contrast transmission electron microscopy (J. S. Teppema, unpublished observation). For that matter, SEM studies do not allow the observation of fimbriae (or pili) on bacterial surfaces, the resolution being too low. From the present study it can be concluded that hemagglutinating activity, as measured in vitro, does not appear to be a necessary prerequisite enabling certain strains of *V. cholerae* to adhere in vivo, at least in the animal model used. This conclusion is in agreement with that of Bhattacharjee and Srivastava (5).

In the present study, hemagglutinating activity, as determined in vitro, was used as an indicator of adherence capacity. However, other *V. cholerae* factors or surface antigens might be responsible for adherence (30). Several cell-bound and soluble hemagglutinins have been recognized (11, 23, 26, 30), and Finkelstein et al. (10) recently reported that *V. cholerae* produces a single polymeric protein that combines hemagglutinating properties with other functions, i.e., it participates in attachment to the gut epithelium, it mediates detachment of the epithelium and, being a prote-

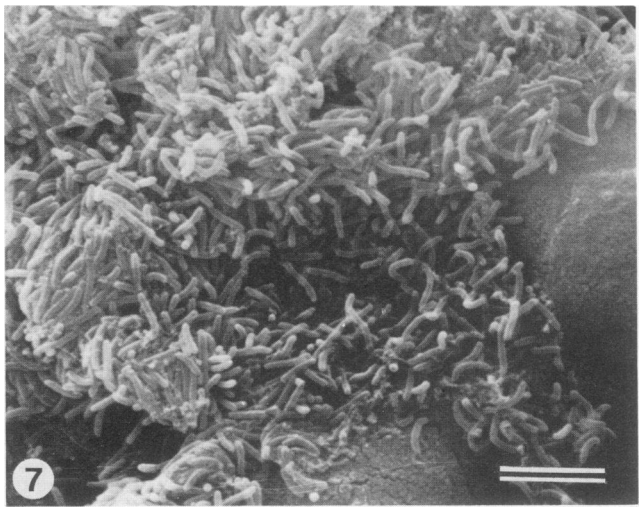

FIG. 7. Large colony of C21 bacteria covering many intestinal epithelial cells at 9 h p.i. Bar, 5 μm.

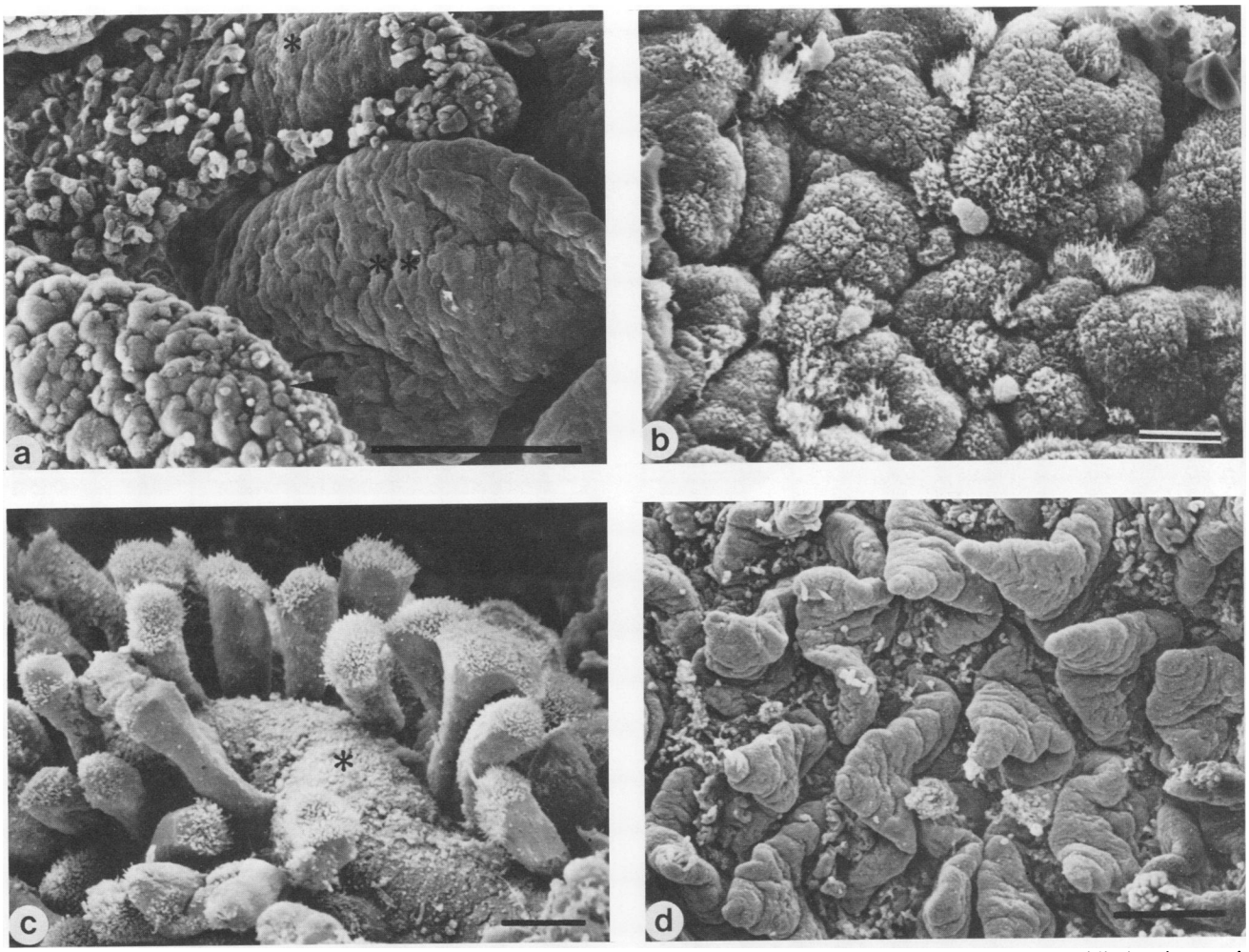

FIG. 8. Variations in the extent of the damage of the villous epithelium at 12 h after C5 or C21 inoculation. (a) Villi showing partial (asterisk) or total (double asterisks) denudation shown next to part of a villus with a greatly roughened epithelium still present (arrowhead). (b) Extremely roughened epithelium with many microvillous irregularities. (c) Groups of epithelial cells have fallen off, leaving a bare basal lamina (asterisk) with single cells still present. (d) A group of totally denuded, shrunken villi with loose aggregates of epithelial cells scattered in between. Bars, 100 μm (a and d) and 10 μm (b and c).

ase, it hydrolyzes fibronectin and mucin and may be responsible for the cleavage of the A subunit of the cholera enterotoxin. Very recently, Pierce et al. (34) showed that cholera toxin contributes significantly to the mucosal adherence of *V. cholerae*. Furthermore, a series of studies by Freter et al. (14, 15, 17) suggested that chemotaxis plays a role in the association of *V. cholerae* with the intestinal mucosa. Moreover, hydrophobic interactions to overcome the repulsive electrostatic forces might be involved, resulting in specific binding of adhesins to surface receptors on epithelial cells (18). Recently, Kabir and Ali (29) demonstrated the presence on the surfaces of several *V. cholerae* isolates of nonspecific hydrophobic and ionic factors, different from specific hemagglutinating factors, which may also be responsible for adherence.

Motility is also regarded as a factor contributing to virulence, in terms of promoting adherence (2, 16, 19, 32, 42) as well as in terms of facilitating penetration of the intestinal mucous blanket (27, 37), and the locomotion of motile bacteria is believed to be guided by chemotactic stimuli (15, 17). However, it has also been suggested that the property of motility is neither necessary nor sufficient for adherence in vitro (2, 28). Although motility obviously favors penetration through the mucous layer and hence adherence, the possibility suggested by the present study that either chemotaxis alone or chemotaxis combined with forces other than motility might afford to nonflagellated strains of *V. cholerae*, such as C21, the ability to arrive at the adhesion site cannot be excluded. The possibility that in vivo synthesis of flagella might occur, as suggested by Freter (14), can be rejected in the present study with the animal model used, since no flagellated C21 vibrios were detected with certainty in the course of our SEM study.

Several studies in the literature (e.g., 15, 17, 20, 27, and 37) emphasize the possible interaction of vibrios with the intestinal mucous blanket. However, there is no agreement on a possible role of the mucus, either as a barrier (15, 17, 27, 37) or as a niche (20), in the mechanism of cholera pathogenesis. Unfortunately, the presence of the mucous layer would hamper SEM observations of the villous epithelium lying beneath it, so it seemed appropriate to study both unwashed and washed intestinal tissue samples. However, from the present study no conclusions could be drawn on the involvement of the mucus. Methods to retain and stabilize

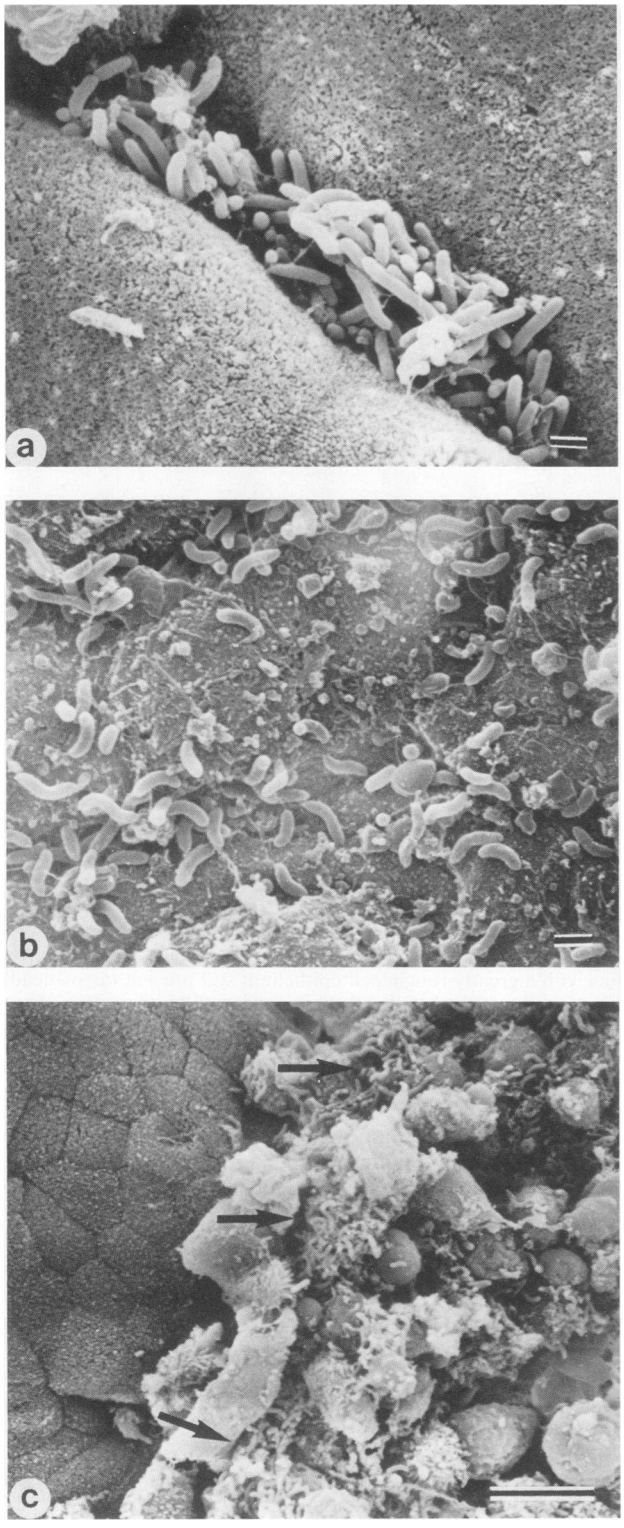

FIG. 9. C5 and C21 vibrios at 12 h p.i. (a) C21 colony in a villous fold; (b) C5 vibrios on bare villous basal lamina; (c) numerous C5 vibrios (arrows) in a cellular aggregate consisting of extruded epithelial cells alongside relatively normal villous epithelium. Bars, 1 μm (a and b) and 10 μm (c).

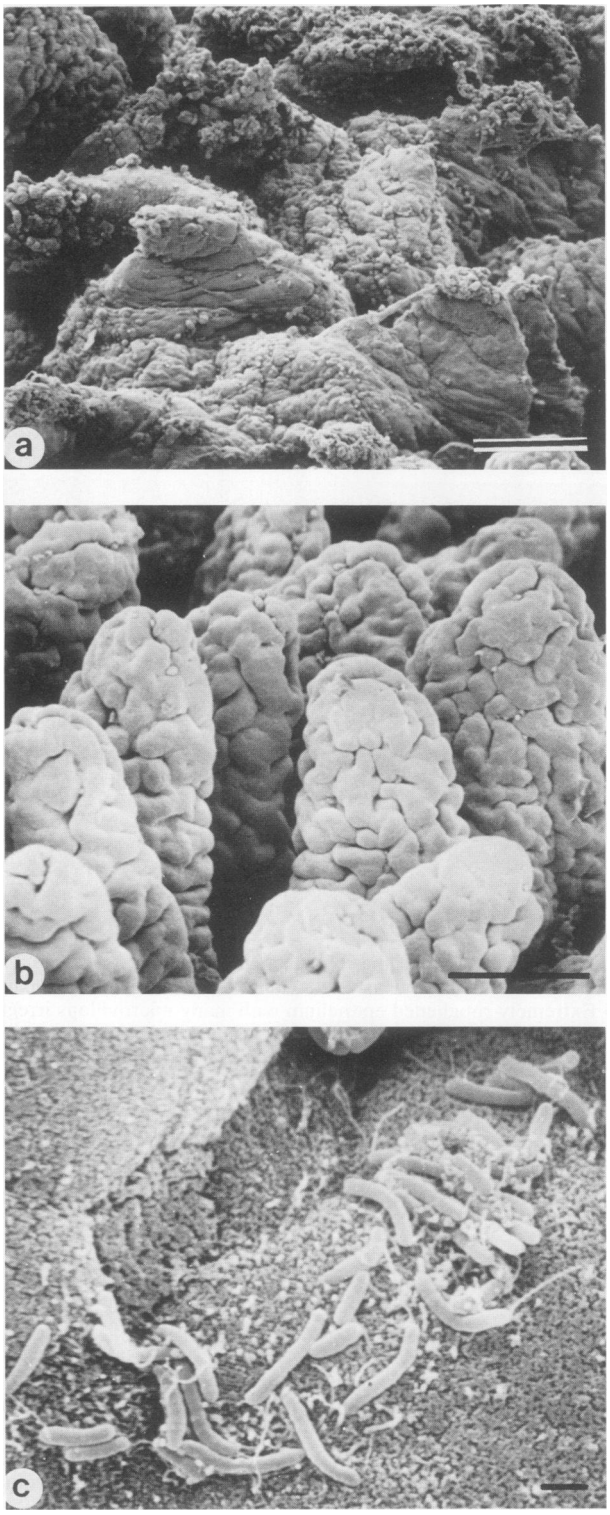

FIG. 10. Positive controls at 12 h p.i. (a) Small intestinal villi with blunt shape, a very roughened appearance or extensive denudation, and loose cellular aggregates, from a loop inoculated with purified cholera toxin; (b) survey of almost normal villi as present in loops inoculated with *V. cholerae* 35A3; (c) moderately colonizing strain 35A3 vibrios. Bars, 100 μm (a and b) and 1 μm (c).

the mucous layer in future experiments, e.g., as suggested by Rozee et al. (36), might provide more information on the role of the mucus.

The few SEM studies focusing on the sequence of the pathological changes of the small intestine during experimental *V. cholerae* infections in animal models (20, 32) did not mention the denudation of the villi, as observed in the present study. However, the extrusion and denudation phenomena observed at 12 h p.i. in C5- and C21-infected loops might be part of a normal pathophysiological process, in view of the fact that these phenomena were absent in strain 35A3-inoculated loops at 12 h p.i. Moreover, initial signs of extrusion of swollen epithelial cells other than that expected at the tips of villi (35) were already apparent at earlier stages. *V. cholerae* non-O-1 clinical isolates have been reported to cause the destruction of intestinal villi (22, 31). Villous denudation was also noted in intestinal biopsies from humans with cholera by Asakura et al. (1); according to these authors, Virchow had already described the phenomenon in intestinal lesions caused by cholera in 1879. However, the denudation concept seems controversial, other authors (E. J. Gangarosa, A. Takeuchi, and H. Sprinz, Am. J. Dig. Dis. **19**:291, 1974; D. G. Sheanan, Am. J. Dig. Dis. **19**: 292–293, 1974), calling it a preparatory or an autolytic artifact. In our opinion, however, the high hydrostatic pressure from the excessive fluid accumulation induced by the release of enterotoxin was responsible for the serious morphological damage, as also suggested by the findings in the cholera toxin-injected loops. Less artificial experimental animal models, such as the RITARD model of Spira et al. (38), our recently described modifications of the RITARD model (21), or the oral inoculation model described by Cray et al. (7), may be more appropriate for the study of the later events in cholera.

The reported observation that vibrios show a roughened appearance at 9-h p.i., in contrast to the smooth appearance of the bacteria at 3, 6, and 12 h p.i., might represent the morphological manifestation of the excretory mechanism of *V. cholerae* described in the literature (6). The timing fits well with the serious fluid accumulation in loops and the subsequently observed massive damage of the villous epithelium, assuming that large amounts of enterotoxin are being released by this mechanism at about this time.

In conclusion, more and intensive studies with suitable animal models are needed to evaluate further the factors of *V. cholerae* really responsible for association with and adherence to the small intestinal surface. Future studies will focus on the pathogenicity of *V. cholerae* strains in a modified removable intestinal tie model described earlier (21).

ACKNOWLEDGMENTS

We thank W. H. Jansen for his expert technical assistance and fruitful discussions, H. Gielen and J. H. L. Westendorp for skillful technical assistance in surgical procedures and animal care, and H. Kleintjes for photographic assistance. We are grateful to Ria Struys for excellent secretarial help.

LITERATURE CITED

1. **Asakura, H., A. Morita, T. Morishita, M. Tsuchiya, H. Fukumi, M. Ohasi, C. Uylangco, and A. Castro.** 1973. Pathologic findings from intestinal biopsy specimens in human cholera. Am. J. Dig. Dis. **18**:271–278.
2. **Attridge, S. R., and D. Rowley.** 1983. The role of the flagellum in the adherence of *Vibrio cholerae*. J. Infect. Dis. **147**:864–872.
3. **Barua, D., and S. N. Chatterjee.** 1966. Electron microscopy of El Tor vibrios. Indian J. Med. Res. **52**:828–830.
4. **Beachey, E. H.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:325–345.
5. **Bhattacharjee, J. W., and B. S. Srivastava.** 1978. Mannose-sensitive haemagglutinins in adherence of *V. cholerae* El Tor to intestine. J. Gen. Microbiol. **107**:407–410.
6. **Chatterjee, S. N., and J. Das.** 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. J. Gen. Microbiol. **49**:1–11.
7. **Cray, W. C., Jr., E. Tokunaga, and N. F. Pierce.** 1983. Successful colonization and immunization of adult rabbits by oral inoculation with *Vibrio cholerae* O1. Infect. Immun. **41**: 735–741.
8. **De, S. N., and D. N. Chatterjee.** 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. **66**:559–562.
9. **Evans, D. G., D. J. Evans, Jr., and S. L. Gorbach.** 1973. Identification of enterotoxigenic *Escherichia coli* and serum antitoxin activity by the vascular permeability factor assay. Infect. Immun. **8**:731–735.
10. **Finkelstein, R. A., M. Boesman-Finkelstein, and P. Holt.** 1983. *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F. M. Burnet revisited. Proc. Natl. Acad. Sci. USA **80**:1092–1095.
11. **Finkelstein, R. A., and L. F. Hanne.** 1982. Purification and characterization of the soluble hemagglutinin (cholera lectin) produced by *Vibrio cholerae*. Infect. Immun. **36**:1199–1208.
12. **Foo, E. S. A., M. Riganti, E. Pongponratn, and W. Chaicumpa.** 1981. Time of maximum colonization by *Vibrio cholerae* El Tor in 5-6 day old mice as an experimental cholera model. Southeast Asian J. Trop. Med. Public Health **12**:609–610.
13. **Foster, J. W., and D. M. Kinney.** 1985. ADP-ribosylating microbial toxins. Crit. Rev. Microbiol. **11**:273–298.
14. **Freter, R.** 1980. Association of enterotoxigenic bacteria with the mucosa of the small intestine: mechanism and pathogenic implications. Nobel Symp. **43**:155–170.
15. **Freter, R., B. Allweiss, P. C. M. O'Brien, S. A. Halstead, and M. S. Macsai.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vitro studies. Infect. Immun. **34**:241–249.
16. **Freter, R., and G. W. Jones.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with intact mucosal surfaces. Infect. Immun. **14**:246–256.
17. **Freter, R., P. C. M. O'Brien, and M. S. Macsai.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. Infect. Immun. **34**:234–240.
18. **Gaastra, W., and F. K. de Graaf.** 1982. Host-specific fimbrial adhesins of nonadhesive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46**:129–161.
19. **Guentzel, M. N., and L. J. Berry.** 1975. Motility as a virulence factor for *Vibrio cholerae*. Infect. Immun. **11**:890–897.
20. **Guentzel, M. N., L. Field, G. T. Cole, and L. J. Berry.** 1977. The localization of *Vibrio cholerae* in the ileum of infant mice. Scanning Electron Microsc. **1977/II**:275–282.
21. **Guinée, P. A. M., W. H. Jansen, and P. W. J. Peters.** 1985. *Vibrio cholerae* infection and acquired immunity in an adult rabbit model. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **259**:118–131.
22. **Gyobu, Y., H. Kodama, H. Uetake, and S. Katsuda.** 1984. Studies on the enteropathogenic mechanism of non-O1 *Vibrio cholerae* isolated from the environment and fish in Toyama prefecture. Microbiol. Immunol. **28**:735–745.
23. **Hanne, L. F., and R. A. Finkelstein.** 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36**:209–214.
24. **Hansson, H.-A., S. Lange, and I. Lönnroth.** 1984. Internalization *in vivo* of cholera toxin in the small intestinal epithelium of the rat. Acta Pathol. Microbiol. Immunol. Scand. Sect. A **92**:15–21.
25. **Holmgren, J., and A.-M. Svennerholm.** 1983. Cholera and the immune response. Prog. Allergy **33**:106–119.
26. **Jones, G. W.** 1980. The adhesive properties of *Vibrio cholerae* and other vibrio species, p. 219–249. *In* E. H. Beachey (ed.),

Bacterial adherence. Receptors and recognition, series B, vol. 6. Methuen Inc., New York.
27. **Jones, G. W., G. D. Abrams, and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immun. **14**:232–239.
28. **Jones, G. W., and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. **14**:240–245.
29. **Kabir, S., and S. Ali.** 1983. Characterization of surface properties of *Vibrio cholerae*. Infect. Immun. **39**:1048–1058.
30. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47**:510–550.
31. **Madden, J. M., W. P. Nematollahi, W. E. Hill, B. A. McCardell, and R. M. Twedt.** 1981. Virulence of three clinical isolates of *Vibrio cholerae* non-O-1 serogroup in experimental enteric infections in rabbits. Infect. Immun. **33**:616–619.
32. **Nelson, E. T., J. D. Clements, and R. A. Finkelstein.** 1976. *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. Infect. Immun. **14**: 527–547.
33. **Ottow, J. C. G.** 1975. Ecology, physiology and genetics of fimbriae and pili. Annu. Rev. Microbiol. **29**:79–108.
34. **Pierce, N. F., J. B. Kaper, J. J. Mekalanos, and W. C. Cray, Jr.** 1985. Role of cholera toxin in enteric colonization by *Vibrio cholerae* O1 in rabbits. Infect. Immun. **50**:813–816.
35. **Potten, C. S., and T. D. Allen.** 1977. Ultrastructure of cell loss in intestinal mucosa. J. Ultrastruct. Res. **60**:272–277.
36. **Rozee, K. R., D. Cooper, K. Lam, and J. W. Costerton.** 1982. Microbial flora of the mouse ileum mucous layer and epithelial surface. Appl. Environ. Microbiol. **43**:1451–1463.
37. **Schrank, G. D., and W. F. Verwey.** 1976. Distribution of cholera organisms in experimental *Vibrio cholerae* infections: proposed mechanisms of pathogenesis and antibacterial immunity. Infect. Immun. **13**:195–203.
38. **Spira, W. M., R. B. Sack, and J. L. Froehlich.** 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. Infect. Immun. **32**:739–747.
39. **Srivastava, R., V. B. Sinha, and B. S. Srivastava.** 1980. Events in the pathogenesis of experimental cholera: role of bacterial adherence and multiplication. J. Med. Microbiol. **13**:1–9.
40. **Tweedy, J. M., R. W. A. Park, and W. Hodgkiss.** 1968. Evidence for the presence of fimbriae (pili) on Vibrio species. J. Gen. Microbiol. **51**:235–244.
41. **Verwey, W. F.** 1976. Current concepts of the pathogenesis of cholera infection, p. 311–316. *In* H. Fukumi and Y. Zinnaka (ed.), Symposium on Cholera, Sapporo, Japan, 1976. Proceedings of the 12th Joint Conference on Cholera, US-Japan Cooperative Medical Science Program. National Institute of Health, Tokyo, Japan.
42. **Yancey, R. J., D. L. Willis, and L. J. Berry.** 1978. Role of motility in experimental cholera in adult rabbits. Infect. Immun. **22**:387–392.