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Vibrio cholerae Adherence and Colonization in Experimental Cholera: Electron Microscopic Studies

EDWARD T. NELSON, JOHN D. CLEMENTS, AND RICHARD A. FINKELSTEIN*

Department of Microbiology, The University of Texas Southwestern Medical School, Dallas, Texas 75235

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Colonization of the intestinal epithelium by *Vibrio cholerae* was examined in two model systems, in ligated ileal loops of adult rabbits and in the patent gut of infant rabbits, using both scanning and transmission electron microscopy. Time studies in the adult model showed a lag period of up to 1 h before the attachment of significant numbers of the vibrios. The bacteria appeared initially in small patches on the sides of the villi, predominantly along the transverse furrows. The number of adherent bacteria steadily increased, reaching a maximum between 4 and 7 h, when a dense mat of bacteria several layers thick covered much of the villi. After this time there was a rapid decline in the number of *V. cholerae* bound. By 12 to 16 h only a few bacteria could be seen on the surface of the villi, which had a rough, patchy appearance at these later times. Globular protrusions, with vibrios attached, may play a role in the clearance of bacteria. Colonization and clearance in the patent intestine of the infant rabbit occurred much as in the adult model. However, the bacteria adhered more uniformly and there was no lag in attachment. In both models the majority of bacteria were aligned horizontally with the epithelial surface, but some were attached in an end-on manner, with their flagella extending into the lumen. The bacteria adhered via their surface coats directly to the tips of the microvilli, except for a few vibrios that were partly embedded into the brush border. Some changes in the microvilli occurred as a consequence of the bacterial attachment.

The disease cholera is the consequence of the action of a toxin produced by *Vibrio cholerae* in the small intestine of humans. The toxin has been isolated and characterized, and its mode of action, activation of host adenylate cyclase and consequent hypersecretion of electrolytes and water, is reasonably well understood (10, 11). Less is known, however, of the delivery system: of how the vibrios survive and multiply in the small bowel, which is normally an inimical environment for bacteria.

Because peristalsis and other factors have been demonstrated to be highly efficient in clearance of bacteria from this region (8, 14, 24, 26), the ability to adhere to the intestinal epithelium must play an important role in an organism's ability to colonize the small bowel. Studies using fluorescent antibody (15, 27, 36) have shown that cholera vibrios become closely associated with the intestinal villi. These "bound" vibrios may be more important in producing the disease state than those bacteria free in the lumen (15, 17, 36). However, in these studies few details of the bacterial adhesion can be seen. Transmission electron microscopic studies on cholera (3, 5, 9, 32, 33; T. C. Merrill and H. Sprinz, Fed. Proc. 25:456, 1966) have largely concentrated on possible damage to or changes in the epithelial cells or submucosa rather than on the site or mechanism(s) of attachment of the vibrios themselves.

In this study, we have combined the great depth of field of the scanning electron microscope and the resolving power of the transmission electron microscope to examine *V. cholerae* attachment and colonization in the rabbit small intestine.

MATERIALS AND METHODS

Bacteria. *V. cholerae* strain 3083, El Tor biotype, Ogawa serotype, was the strain used throughout these experiments. The strain has been maintained in the lyophilized state since its isolation in Vietnam (by R.A.F.) in 1964 and has been described previously as being toxigenic (12, 13). It is highly virulent in the infant rabbit model, causing diarrhea by 9 or 10 h and death within 16 h of infection. Stock cultures were kept lyophilized and, upon reconstitution, were maintained on Trypticase soy agar slants at 4°C. For infection, an overnight culture in Trypticase soy broth (BBL) was diluted 1:100 into fresh Trypticase soy broth and grown with shaking at 37°C to mid-log-phase growth. The bacte-

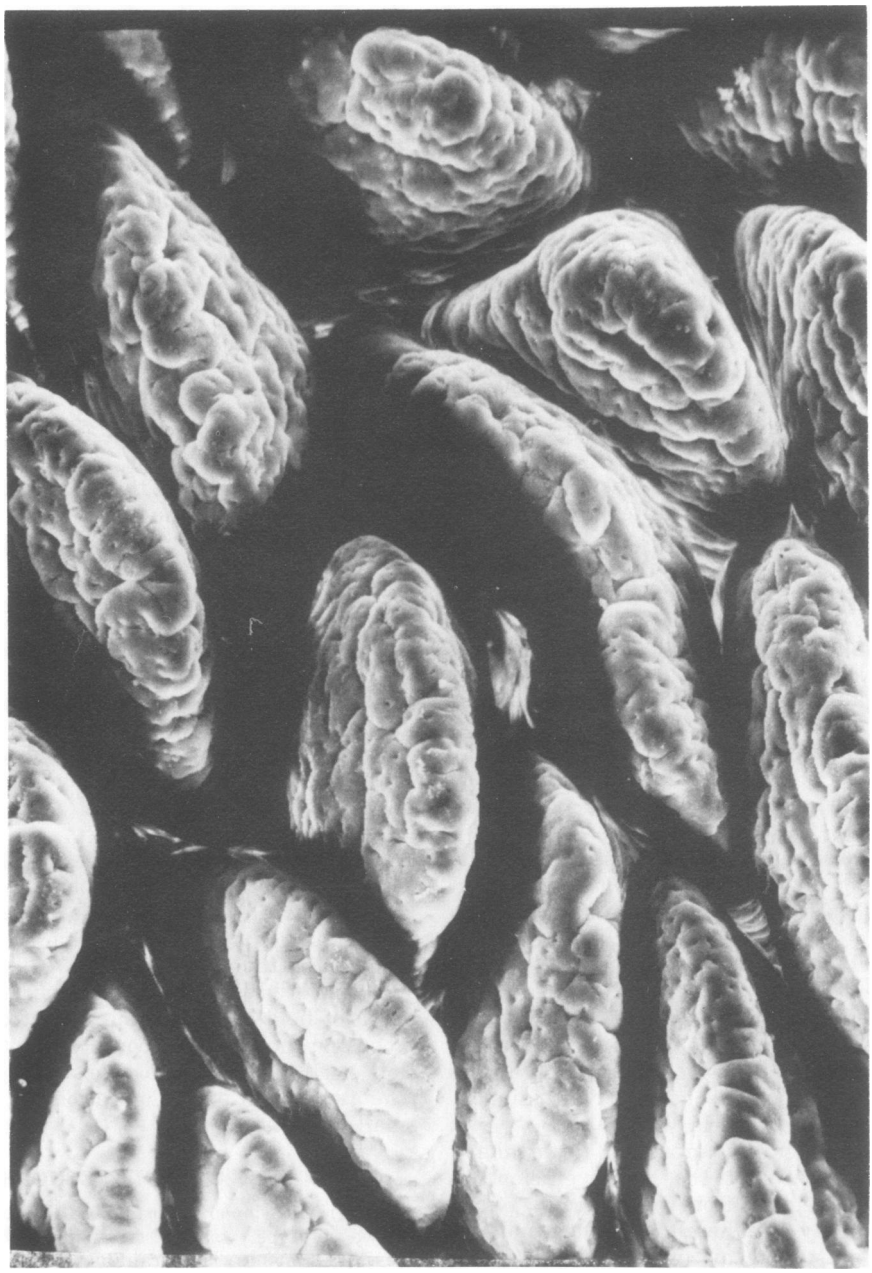

FIG. 1. *Control adult rabbit intestinal loop segment showing villi with transverse furrows and depressions.* ×*400.*

ria were collected by centrifugation, washed with phosphate-buffered saline (PBS), pH 7.4, and resuspended in PBS to the desired concentrations.

Animals. Young adult New Zealand white rabbits were obtained from the Southwestern Medical School Animal Resource Center. New rabbits were kept in separate cages and given food and water ad lib. Only healthy animals were used for the experiments. Five- to 7-day-old infant rabbits were kept with their mothers until just prior to use.

Adult rabbit ileal loop model. Adult rabbits were anesthetized intramuscularly with Inovar-Vet (Pitman-Moore, Washington Crossing, N.J.) and locally with Lidocaine-hydrochloride (Anthony Products Co., El Monte, Calif.). The abdomen was opened, and the small intestine was tied into 6-cm segments

with 2-cm spacer loops between the experimental loops. The large loops were injected with 0.5 ml of bacterial suspension (2×10^8 bacteria/ml). Control (6 cm) loops were injected with 0.5 ml of PBS. Generally, five to seven loops were made per rabbit. For each experiment two rabbits with the samples injected in reverse order were used. For time studies, all the loops were prepared at the initial operation and then injected at various times thereafter. Between operations, the incisions were closed with sutures and surgical skin clips and the rabbits were allowed to rest on their abdomens; otherwise trauma and lung congestion would sometimes result. At the end of the experiment, the rabbits were sacrificed and the intestines were removed immediately. In some experiments the intestine and bacteria were fixed in situ by injecting 1 ml of 2% glutaraldehyde in sterile PBS into the loop 5 min before sacrificing

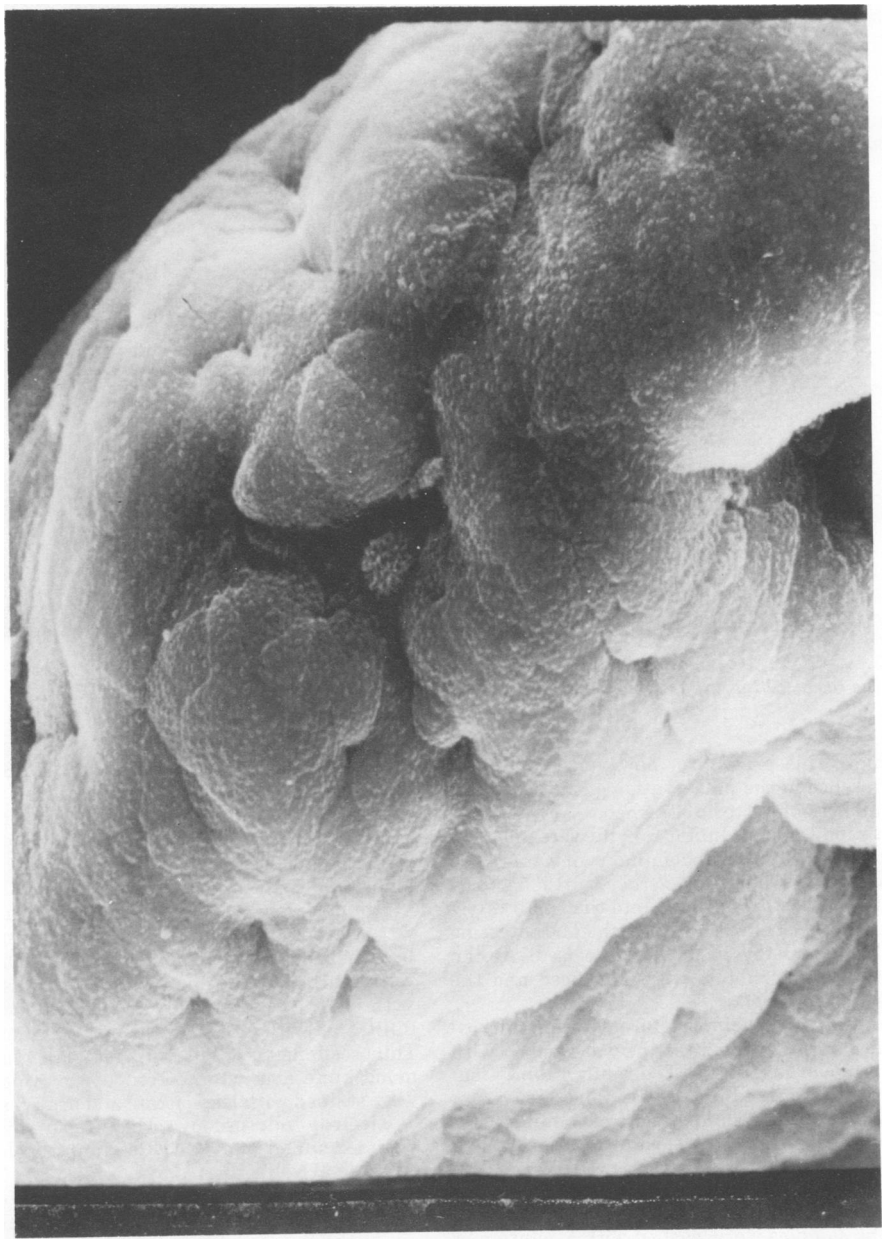

FIG. 2. *Control adult rabbit intestinal villus. Note the absence of indigenous microorganisms and debris.* ×*1,560.*

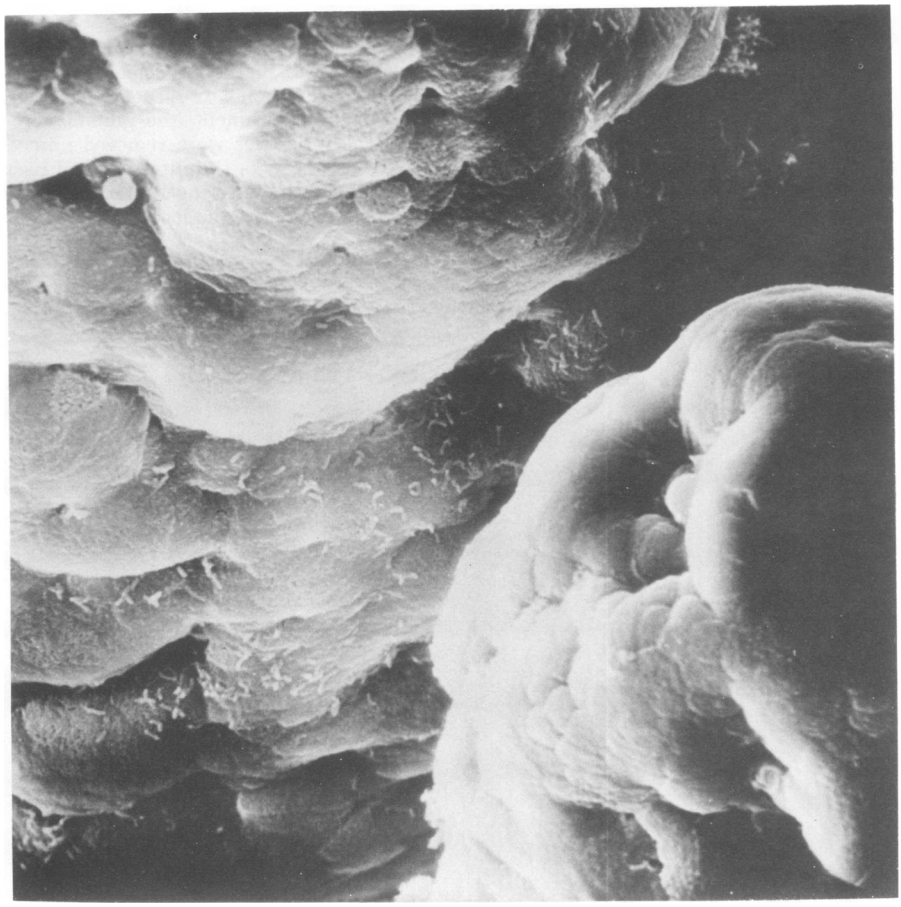

FIG. 3. *V. cholerae attached to adult rabbit intestinal villi 1 h postinfection. The vibrios show a predilection for the areas along the transverse furrows.* ×*1,000.*

the animal. This procedure, however, resulted in little difference in the preservation of the sample or in the numbers of bacteria attached.

Infant rabbit model. Infant rabbits were anesthetized with ether, and the abdominal wall was opened to expose the intestine. *V. cholerae*, 2×10^8 bacteria in 0.5 ml of PBS, were injected into the small intestine immediately below the stomach. The intestine was replaced and the incision was closed. At the desired times, the rabbits were sacrificed and the intestine was removed.

Preparation of specimens for electron microscopy. The exterior of the intestine was washed with PBS to remove any blood. The adult rabbit ileal loops were each opened under PBS, and 3- to 5-mm segments were removed. Segments of the infant intestine were removed from several locations along the intestine. The pieces of intestine were washed by gentle agitation consecutively in four beakers of PBS to remove debris and any bacteria not firmly bound. The samples were washed with a solution of 2% glutaraldehyde in PBS, placed in small vials with 2% glutaraldehyde, and fixed at 4°C for 24 h.

The samples used for scanning electron microscopy were then washed twice with PBS and once with distilled water and dehydrated by a stepwise replacement of the water with acetone. The samples were then dried in a critical-point drying apparatus (Polaron E3000), trimmed, mounted on aluminum stubs, and coated with gold-palladium.

Samples for transmission electron microscopy were washed twice with PBS and then postfixed for 1 h with 1% osmium tetroxide in PBS. The samples were washed four times with PBS, washed twice with distilled water, dehydrated with acetone, and infiltrated with either an Epon or Marglas solution. Thin sections were cut with either a glass or diamond knife and mounted on copper grids. Sections were stained with lead citrate and uranyl acetate.

Electron microscopy. The prepared specimens were examined in a JOEL-35 scanning electron microscope at voltages of 15, 20, and 25 kV. Transmission electron microscopy was performed on either a JOEL-100B or a Philips 301 electron microscope.

RESULTS

Adult rabbit ileal loops. The villi of the control intestinal loops were, after the washing

steps, remarkably free of both bacteria and debris (Fig. 1 and 2). The very few bacteria that were occasionally seen on the surface of the villi were large, thick, rod-shaped organisms, resembling in size and shape the bacteria shown by Savage and Blumershine (35) to colonize the murine stomach. These organisms, which were easily distinguished from *V. cholerae*, are present in moderate numbers in the lumen of the small intestine, as shown in unwashed samples, where clumps of these bacteria were found in the debris that covered and obscured the villi from view. This normal freedom of the surface from bacterial contamination is not entirely due to the natural flushing action and motility of the gut, since even villi from control loops closed for up to 24 h still had no bacteria attached.

The villi themselves had a smooth textured surface, which was interrupted by numerous small holes present from tip to base and also occasional larger cavities generally confined to the upper portion. The small openings probably represent the mouths of goblet cells (1, 30) through which mucus is extruded into the lumen. The larger depressions could be the result of epithelial cells having been shed from the villi into the lumen, since this is thought to occur at the tips of the villi (4). Also seen on the villi were transverse furrows, which may be caused by contraction of the villus (G. L. Waxler, D. P. Olson, and D. E. Spotts, 11th Annu. Meet. Assoc. Gnotobiot., 9-13 June 1974, Guelph, Ont., Canada, cited in reference 20).

We were interested in studying both the location and time course of *V. cholerae* colonization and in the mode of attachment of the bacteria to the surface as seen by the scanning electron microscope. Time studies were performed in which samples were taken as early as 10 min and as late as 24 h after the bacteria were inoculated into the loops. The early samples indicated a lag in the attachment of the vibrios to the intestinal surface. No attached bacteria could be found on the samples taken at 10 and 20 min. Even at times as late as 30 and 40 min postinfection, with extensive searching, only an average of two or three vibrios could be found per villus. It was not until 1 h (Fig. 3) that vibrios could be found with regularity, and even then they appeared unevenly dispersed; one villus might have several hundred attached bacteria, whereas the villus next to it had none. The bacteria tended to occur in patches and showed a predilection for the areas along the transverse furrows or grooves of the villi.

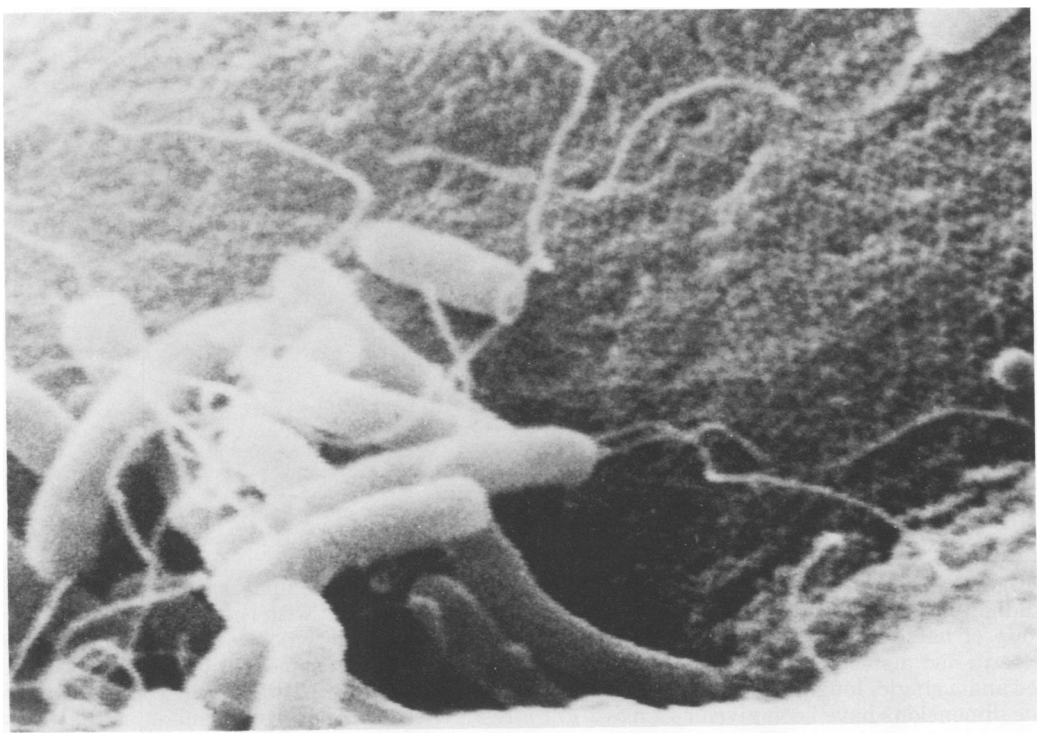

FIG. 4. *V. cholerae adhering to adult rabbit ileal epithelium. Microvilli tips appear as a regular array of closely packed particles.* ×25,000.

FIG. 5. *V. cholerae attached end-on, with flagella extending out into the lumen.* ×*30,000.*

Figures 4 and 5 show higher magnifications of adherent *V. cholerae*. Figure 4 shows a small group of bacteria located near a furrow. The bacteria had a smooth but slightly "fuzzy" surface and a single, long polar flagellum. *V. cholerae* dimensions have been given as 1.5 to 2 μm long and 0.3 to 0.4 μm wide (31). Our measurements, based on comparison with standard latex beads, agreed with these. Some longer bacteria were found which could be in the process of dividing. Also, some of the bacteria appeared shorter and seemed lighter in contrast to the rest. These were attached end-on so that they gave a foreshortened appearance. Occasionally

the vibrios were located in such a position that they could be viewed from a more horizontal plane (Fig. 5). These views confirmed that at least some of the vibrios attach end-on, with the flagella extending out into the lumen. Although this particular micrograph (Fig. 5) does not show clearly from which end of the bacteria the flagella arise, in the majority of cases the flagellum originated at the free end of the bacterium.

The texture of the villus surface can be seen in Fig. 4 and appears to consist of a regular array of closely packed particles. These particles could represent the tips of the microvilli, since our measurement of a 0.07-μm diameter is close to the accepted value of 0.1 μm (38) and they have the same cobblestone appearance as Balcerzak et al. (1) described for rat and human microvilli.

The numbers of vibrios on the intestinal surface steadily increased and reached a maximum between 4 and 7 h (Fig. 6). By this time large patches of bacteria covered much of the villus surface. Higher-magnification examination of these mats of bacteria (Fig. 7) demonstrated no special arrangement of the bacteria and showed that they could be piled several layers thick. Although it was normally difficult to examine the lowest part of the villi and the intervillus space, we were able to examine the villi at the cut edge of the sample and also occasionally isolated complete villi that had been broken off from the sample and found that there was no major difference in the distribution of *V. cholerae* along the side from the top to the base. However, the very tips of the villi were relatively free of large patches of vibrios.

Perhaps the most surprising result of our study was the rapid disappearance of the vibrios from the gut surface after this time. By 10 h the number of *V. cholerae* was much reduced from that seen as late as 6 to 8 h: the number present was about equal to that seen at 2 or 3 h. After 12 h (Fig. 8) only a few vibrios could be

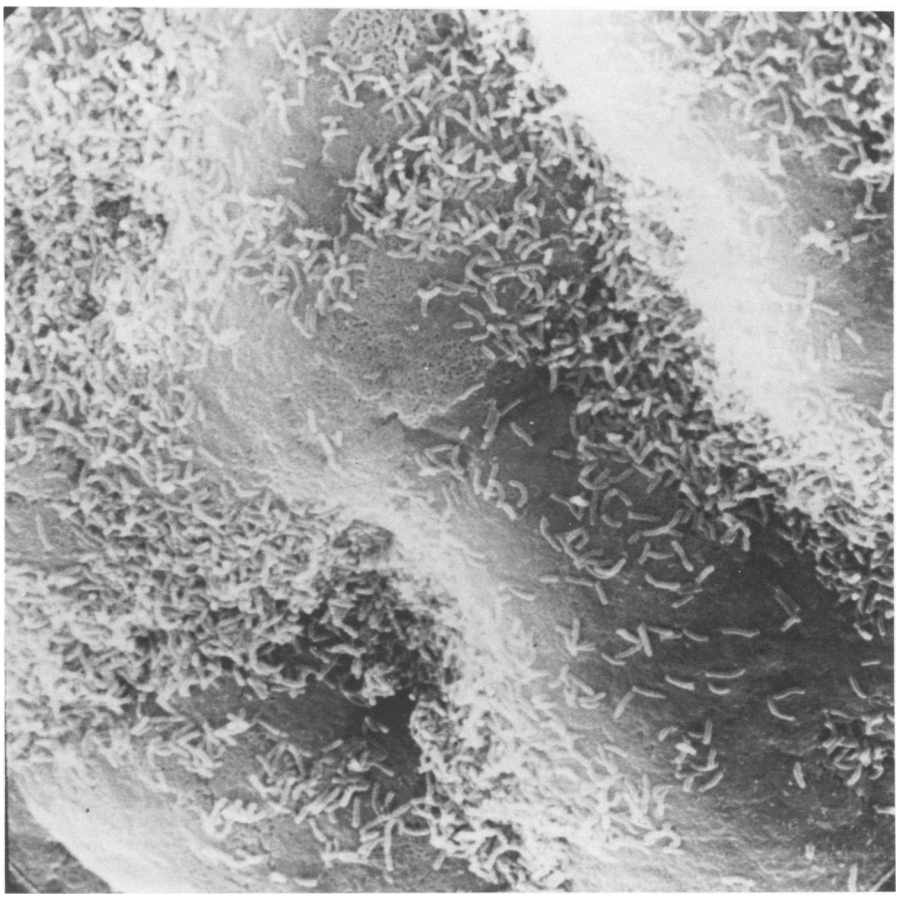

FIG. 6. *A 5-h sample showing large patches of V. cholerae on the adult villus.* ×2,080.

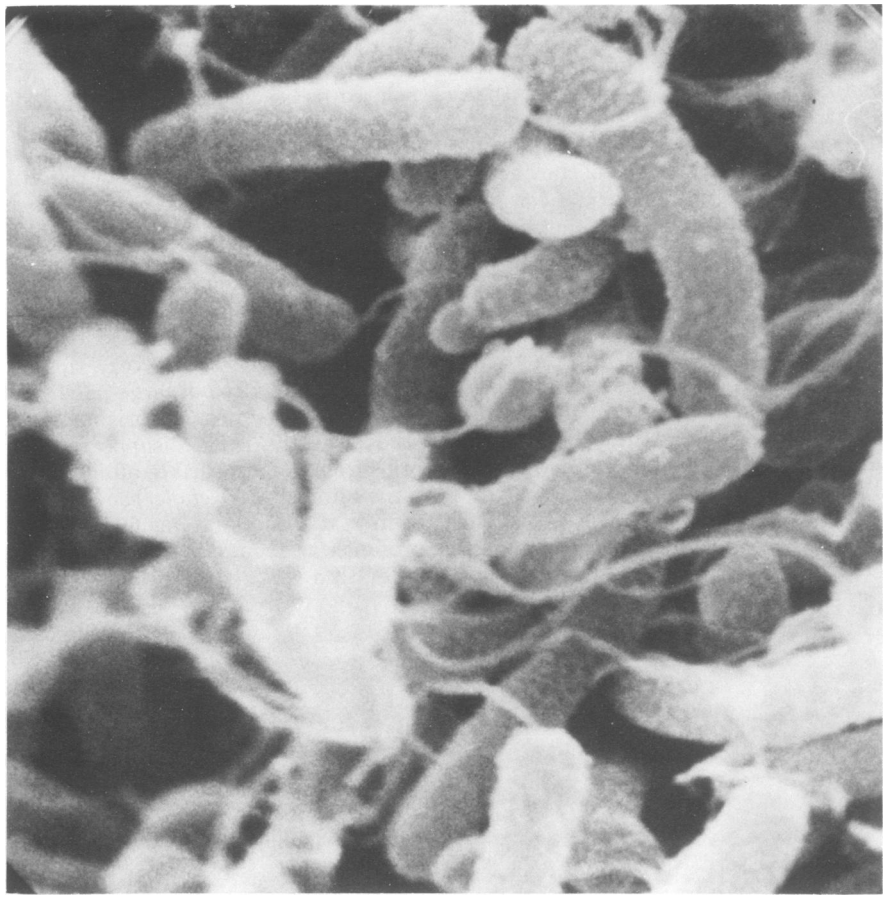

FIG. 7. *Higher magnification of V. cholerae adhering to adult rabbit ileal epithelium at 5 h. Bacteria occur in a mat several layers thick.* ×46,800.

found on the villi (mainly at the tips). The villi from infected loops at these latter times exhibited a rough and patchy appearance in their surface compared with the villi from control loops, which remained unchanged throughout the experiment.

Starting about 7 h, round smooth projections or blebs appeared on the villus surface, originating from the depressions in the surface. These blebs often had bacteria on their surface and seemed to be in the process of being ejected into the lumen. Figure 9 shows a close-up of one of the blebs and its associated bacteria.

Infant rabbits. The villi on the infant rabbit intestinal wall were smaller, thinner, and more pointed than those from the adult and also had shallower grooves and fewer depressions. They were equally as free of adherent bacteria. Colonization of the villi proceeded at a faster rate in this model. As early as 15 min after infection, a few vibrios could be found bound to the villi. By 1.5 h the villi were already covered with bacteria to as great an extent as found in the adults at the peak of adherence. Figure 10 shows that the vibrios attached directly to the surface, as was seen in the adult rabbit model. By 5 h the entire villus surface except the very tip was completely covered with a thick mat of bacteria (Fig. 11 and 12). In the infant rabbit the *V. cholerae* more evenly covered the surface and did not occur in patches as they did in the adult.

Again, as in the adult model, clearance of the bacteria from the surface occurred. In the infant gut this phenomenon occurred at a faster rate. By 9 h most of the bacteria had been removed (see Fig. 13). Also, changes in the surface were noted, mainly an increase in the number of projections, especially at the tips of the villi, and a rough surface appearance. Some samples at the later times exhibited a "fuzzy"

layer on their surface (Fig. 14), which looked much like the covering of bacteria seen at earlier times but at higher magnification (Fig. 15) was shown instead to be due to elongated individual microvilli. A bacterium that illustrates the differences in size is located at the bottom of the picture.

Transmission electron microscopy. We had hoped to find bacteria at early times shortly after their attachment to the villi, but the scarcity of bacteria led us to examine the later times. Figure 16 shows a cross section of a *V. cholerae* adhering directly to the edge of the brush border, its outer membrane in contact with the surface of the host microvilli. Figure 17 shows a longitudinal cross section of a bacterium in contact with the surface and also a bacterium attached more end-on. The body of the organism is curving away from the picture, causing the lower part of the cell envelope to seem to dissolve. The dark-staining granules seen are probably some form of lipid storage granules, since they were only seen in the bacteria from samples postfixed with osmium and not in samples fixed with glutaraldehyde alone but otherwise treated in the same manner. Also seen occasionally, but not shown here, were bacteria that had partially penetrated into the microvilli to varying degrees but never to the point of reaching the base of the microvilli. These bacteria were generally attached end-on. At these later times, in addition to single cells, we also found, as expected from scanning electron microscopy, large masses of organisms adjacent to the surface (Fig. 18). These groups generally seemed to be aligned in the same general direction, although this had not seemed to be the case from the scanning electron microscope studies (see Fig. 7). In with these groups of bacteria there was always a large amount of

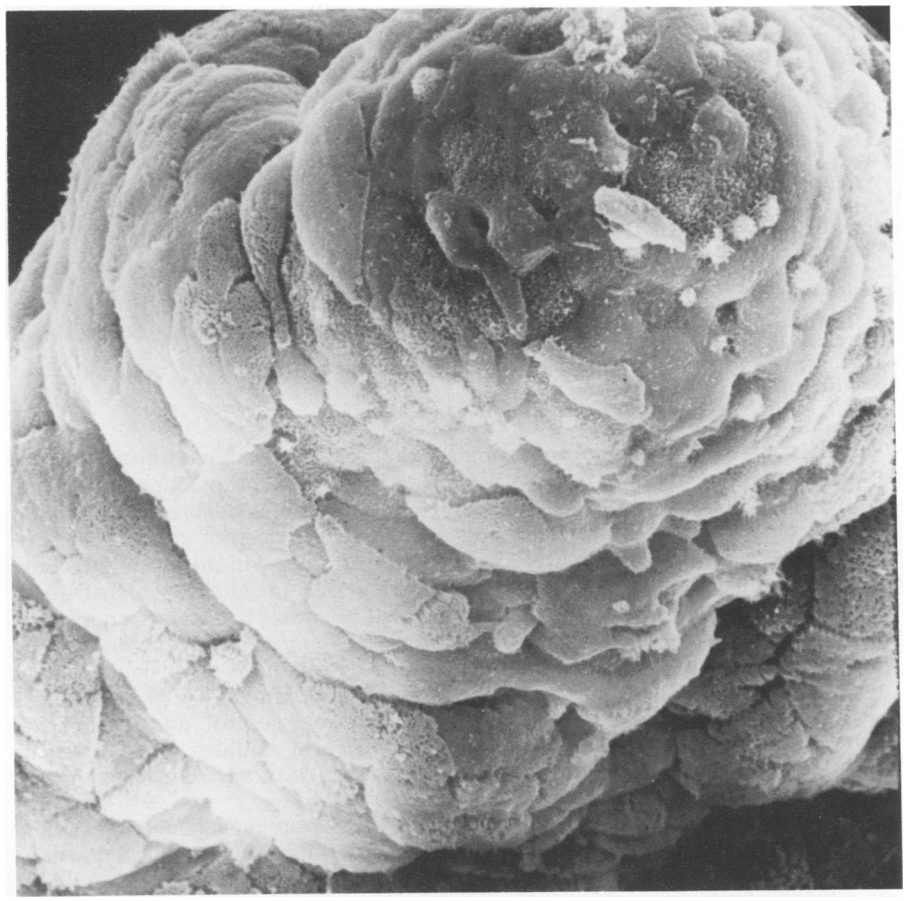

FIG. 8. *Adult rabbit villus showing almost complete absence of vibrios after 12 h of incubation. Note rough, patchy appearance of villus surface.* ×2,300.

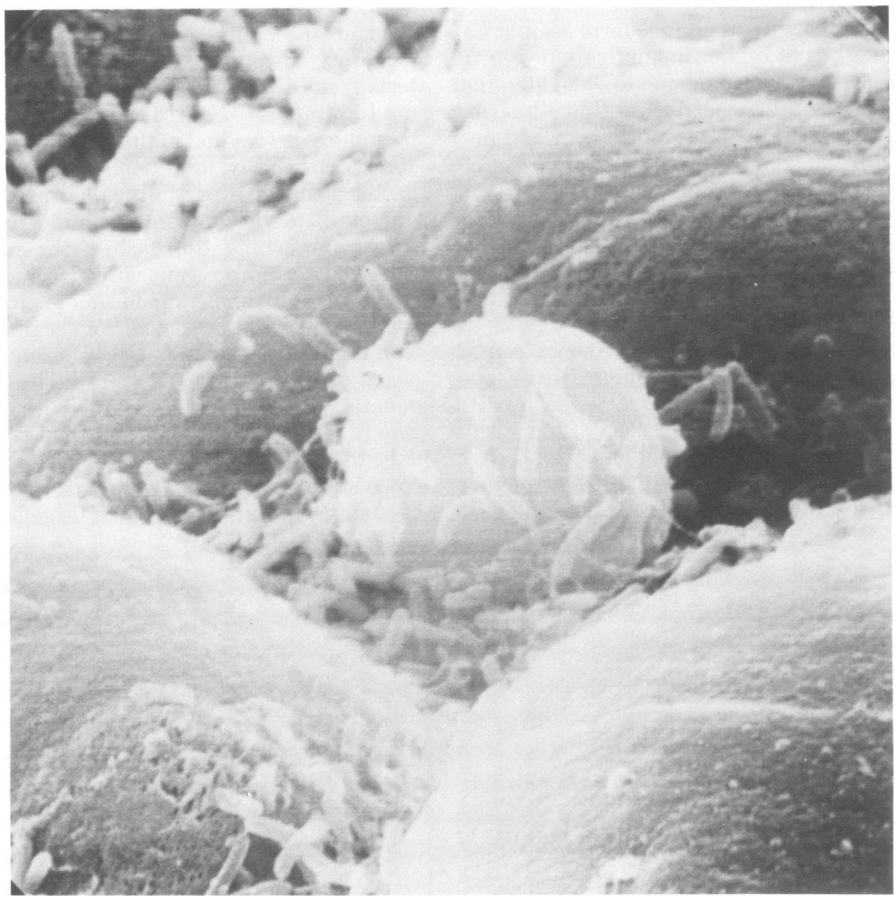

FIG. 9. *High magnification of one of the globular protrusions seen after 7 h of infection. Note the bacteria adhering to its surface.* ×6,500.

debris, mostly consisting of membrane-bound, empty vesicles and what appeared to be fragments of microvilli. Many of the vesicles appear to be formed from the microvilli or their fragments (see arrows). Some of the debris might also arise from dead bacteria. The changes seen in the microvilli near the bacteria (elongation, fragmentation, and vesicle formation) were not seen in control sections.

DISCUSSION

There has been much interest lately in bacterial adhesion to and colonization of the various surfaces of the body and an increasing recognition of the importance of these events in bacterial disease. The scanning electron microscope has made it possible to examine these surfaces and their attached bacterial populations more directly than the light microscope or transmission electron microscope allowed. Our studies of *V. cholerae* adhesion and colonization in the rabbit intestine have uncovered several interesting aspects of infection not shown before.

One of the models selected for study was the ligated ileal loop of adult rabbits. This model was introduced in the early 1900s but not extensively used until its revival by De and Chatterje (6). The model has proven to mimic the effects of *V. cholerae* infection on the human gut rather well and is extensively used as an assay for enterotoxin (2, 10). We found this model to be extremely useful, and it had the advantage that several samples or time periods could be examined with adjacent loops in the same animal.

In this system we noted a lag of about 30 min in the attachment of vibrios to the villi. After this time, irregular patches of vibrios appeared on the villi. This lag seen in attachment was puzzling, since in vitro experiments (unpublished data) involving *V. cholerae* adhesion to erythrocytes, to isolated intestinal cells, and

even to slices of intestine indicate that binding can occur very rapidly. This difference between in vitro and in vivo systems could be due to several factors. One possibility is that there is some barrier in the intact gut that the bacteria must first penetrate to reach the epithelial surface. A likely candidate is the mucus material that overlies the tips of the villi. In a recent study, Schrank and Verwey (36) concluded that the mucus layer might present a barrier that could prevent or slow the penetration of vibrios into the intervillous spaces. Although our studies were concerned with those vibrios directly associated with the epithelial surface, some un-

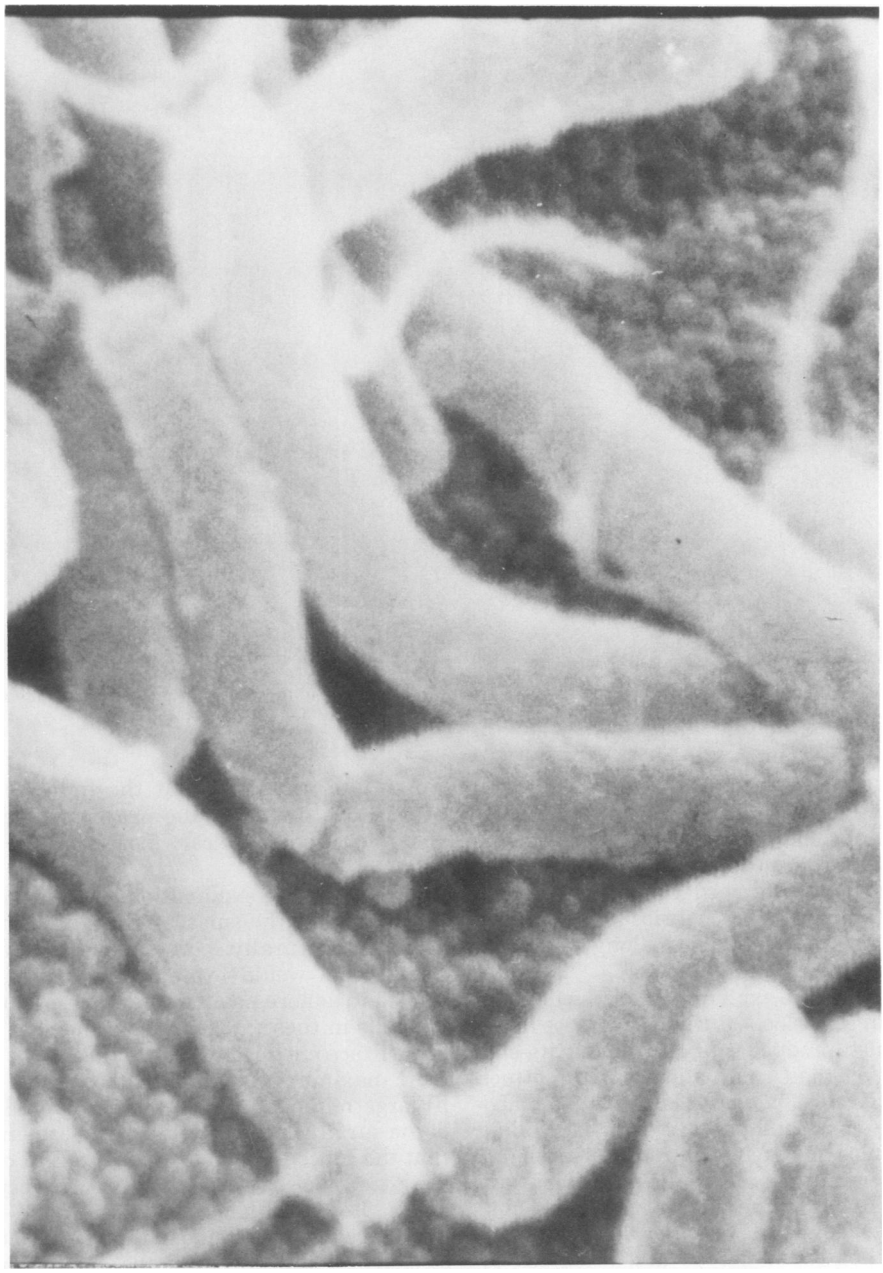

FIG. 10. *V. cholerae adhering to the microvilli tips of the epithelium surface. A dividing bacterium is present in the center.* ×60,000.

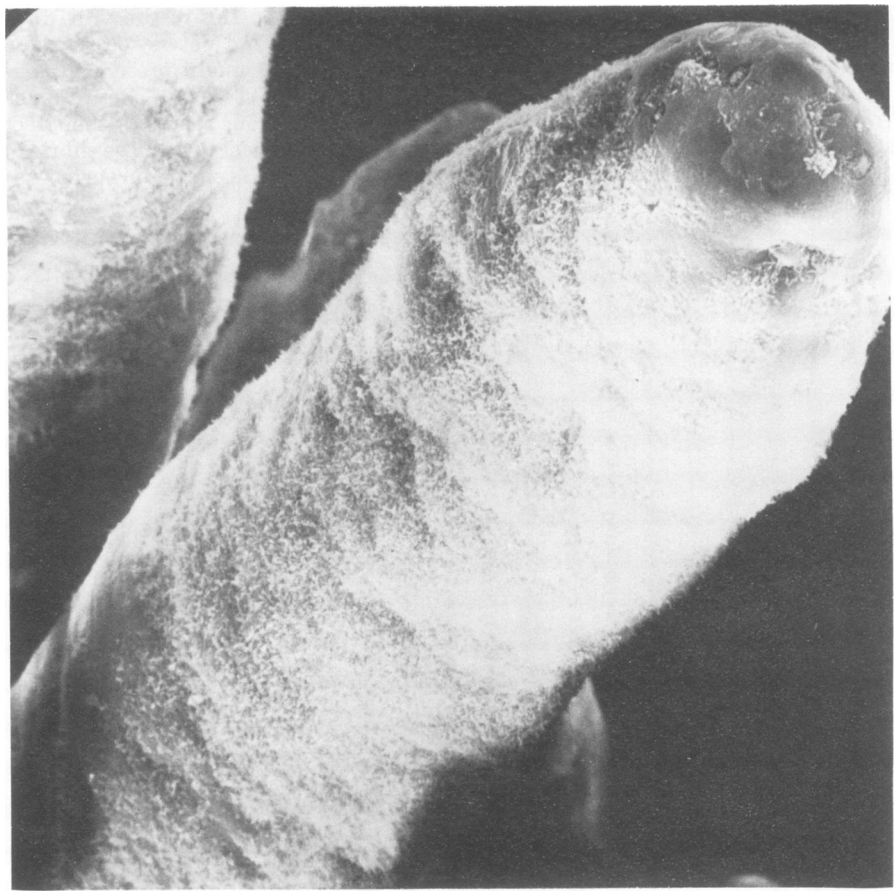

FIG. 11. *A 5-h sample from an infected infant. The vibrios almost completely cover the villus except for the tip.* ×650.

washed samples were also examined (not shown). The mucus existed as a lacelike layer of strands of mucus, lying over the tips of the villi, which did not extend into the intervillus space, much as described by Florey (14). In infected samples many vibrios were always found caught in these strands, along with other bacteria and much debris.

Another possible explanation for the delay in adhesion in adult loops is that the vibrios in the lumen must produce sufficient quantities of some product that would allow them to attach to the surface. *V. cholerae* is known to elaborate many extracellular products, including such enzymes as mucinases and proteases (21, 28, 34). It is interesting that the bacteria are found in patches on the surface instead of being evenly spread over it, even at early times when these patches could not be accounted for by local multiplication of the organisms. This raises the possibility that the first vibrios to attach to a site affect the nearby surface such that other bacteria can more readily attach there.

An alternative explanation for the patches of vibrios is that the surfaces of the villi in these areas are naturally more amenable to attachment. This would explain why the bacteria tended to adhere near the transverse grooves if the cells in these areas had a somewhat different surface chemistry or structure.

At maximum colonization (between 4 and 7 h) large numbers of *V. cholerae* covered much of the surface of the intestine. One can envision that the concentration of enterotoxin at or near the patches of bacteria would reach very high levels. Recent studies (7, 37, 39) have shown that the cells on the side of the villi react more strongly to the effects of the toxin than the crypt cells, thus reversing the formerly held

theory that the crypt cells were preeminently involved. Since the bacteria were found down the side of the villi from tip to base, a large area of the surface would thus be affected by the toxin. It may not be coincidence that the loops become positive with fluid between 4 and 5 h, about the same time the bacteria are reaching their maximum numbers on the surface. However, it must be pointed out that a lag in fluid production is seen even when large amounts of purified toxin are injected into ileal loops.

It was interesting to find that the bacteria in infant rabbits did not exhibit a lag in adhesion and colonized the surface faster. This and the fact that the vibrios reached even higher levels on the infant intestinal epithelium could partly explain why infant rabbits are more susceptible to experimental infection in the patent gut than are adult rabbits.

Another point that indicates how much more susceptible the infant gut surface is to colonization than is that of the adult is that, in the adult ligated ileal loop model, one of the important aspects of host defense in the gut, peristalsis (8, 24), was compromised, whereas in the infant any bacteria unable to readily attach would tend to be rapidly swept out of the gut.

Knop and Rowley (25) have shown that the antibacterial mechanisms in the infant mouse are less effective than are those in the adult mouse. It is possible that natural differences in susceptibility of the intestinal surface to bacterial attachment could have a role in the different morbidity rates of human infants and adults for such enteric diseases as cholera and enteropathogenic *Escherichia coli* diarrhea. It has generally been presumed that these differences are due to the development of specific or nonspecific antibody; however, this has never been conclusively established.

Another difference seen between infection in the adult and infant rabbit systems was in the

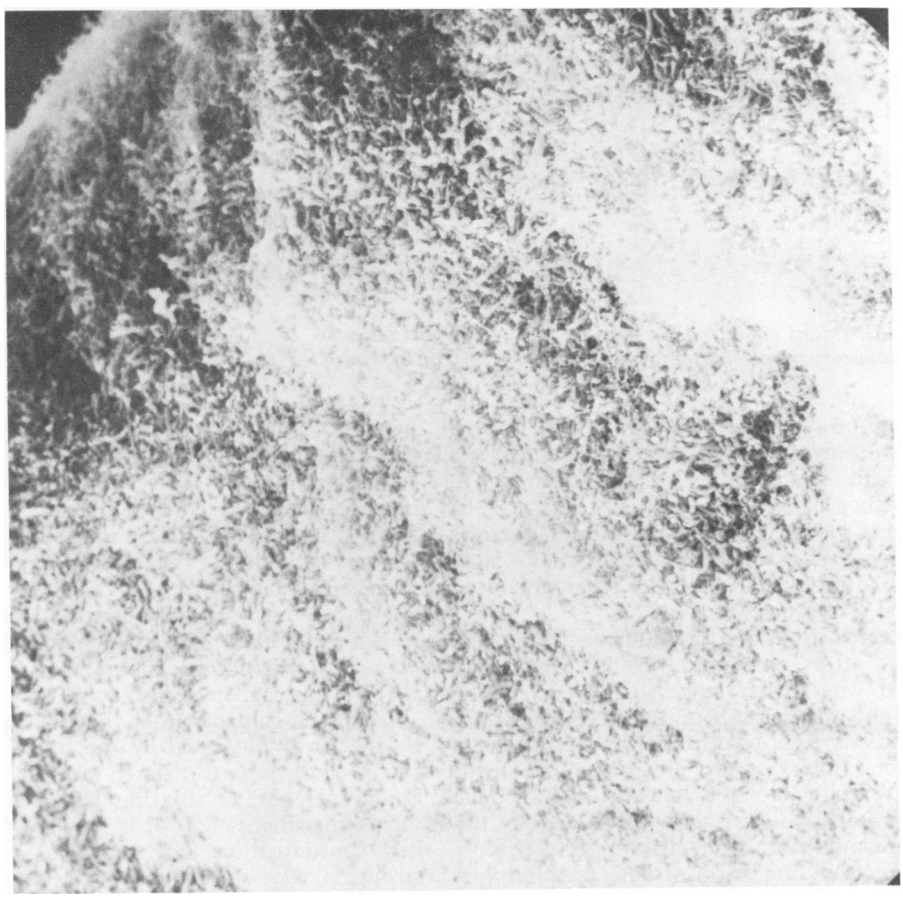

FIG. 12. *Higher magnification of vibrios attached to infant rabbit intestinal villus 5 h postinfection.* ×*1,950.*

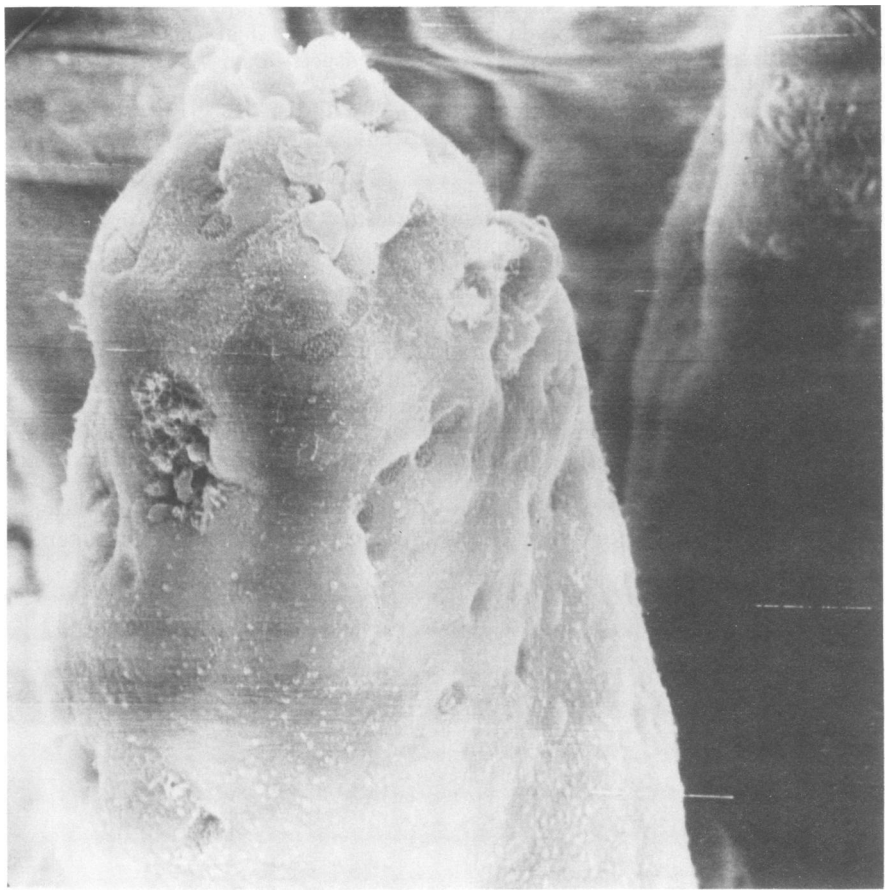

FIG. 13. *Infant rabbit villus showing almost complete absence of vibrios after 9 h of incubation. The surface appears rough, and a protrusion can be seen at the tip.* ×1,170.

distribution of bacteria. In the infant, the vibrios were evenly distributed over the surface of the villi (except for the very tip), whereas in adults they occurred in patches. This may be related to the absence of the transverse furrows in the infant or other factors, such as more uniform distribution of surface receptors or absence of inhibitory material.

The rapid decline in the number of adherent vibrios after maximum colonization was reached was rather surprising. We had expected the number of bound bacteria to remain at relatively high levels over the course of the infection. However, this clearance of the villi was a reproducible occurrence in both models.

At present we have little knowledge of the mechanisms involved in this elimination of *V. cholerae* from the surface. One possibility is that the host is directly involved in some clearance mechanism. This might involve the globular protrusions seen during infection which appear to be in the process of carrying bacteria into the lumen. These might be cells; however, we feel it is more likely that they represent mucus being excreted by the goblet cells. It is known that during cholera the mucus cells are actively secreting or empty (3, 9, 32, 33, 40); in fact, this is one of the most consistent findings of electron microscope studies of cholera. It was shown by Florey (14) that mucus secretion plays an important role in removing inert particles such as carbon from the villus surface.

Another explanation for the clearance could be that it is mediated by the bacteria themselves or through the action of their products. Some evidence for this has been found in in vitro experiments (23). It is possible that local high concentrations of their enzymes could destroy the very surface receptors to which the bacteria are bound. This might partly explain the altered appearance of the villi after the bacteria are gone.

Another possibility that we cannot exclude at the present, but consider unlikely, is that the adherent bacteria are being destroyed or lysed on the surface by some type of host antibacterial response. We saw no evidence of such a mechanism in our studies. Even if a significant mobilization of antibody could be generated in such a short time, the secretory antibody would probably possess antiadhesion rather than bacteriolytic properties (18). However, evidence has been presented (26) for an antibody-independent antibacterial mechanism operating on the surface of the mouse mucosa.

The disappearance of bacteria from the surface might explain why, in previous transmission electron microscopic studies of cholera (3, 5, 9, 32; Merrill and Sprinz, Fed. Proc. 25: 456, 1966), it was either stated that few adherent bacteria were seen or none were shown or mentioned in the study. Since these studies, in both human and experimental cholera, took place late in infection, well after the symptoms of the disease were evident, our work would indicate that few, if any, vibrios would be expected to be seen on the brush border. The one transmission electron microscope study that does show a micrograph of *V. cholerae* adhering to the brush border (33) was performed in rabbits at early times (mainly at 6 h) and thus would be expected to show bacteria attached.

In addition to the colonization aspect of infection, we were also interested in the actual adhesion of the vibrios to the epithelial surface. Scanning and transmission studies showed the bacteria to adhere directly via their surface coat to the microvilli tips (Fig. 4, 10, 16). Some transmission micrographs showed a small separation between the brush border and the bacteria (Fig. 17). This may be due to partial removal of the carbohydrate-rich surface layer or glycocalyx (22) during the sample preparation, or it may not have stained well.

Most of the bacteria were adherent horizontally with the surface, but some vibrios, espe-

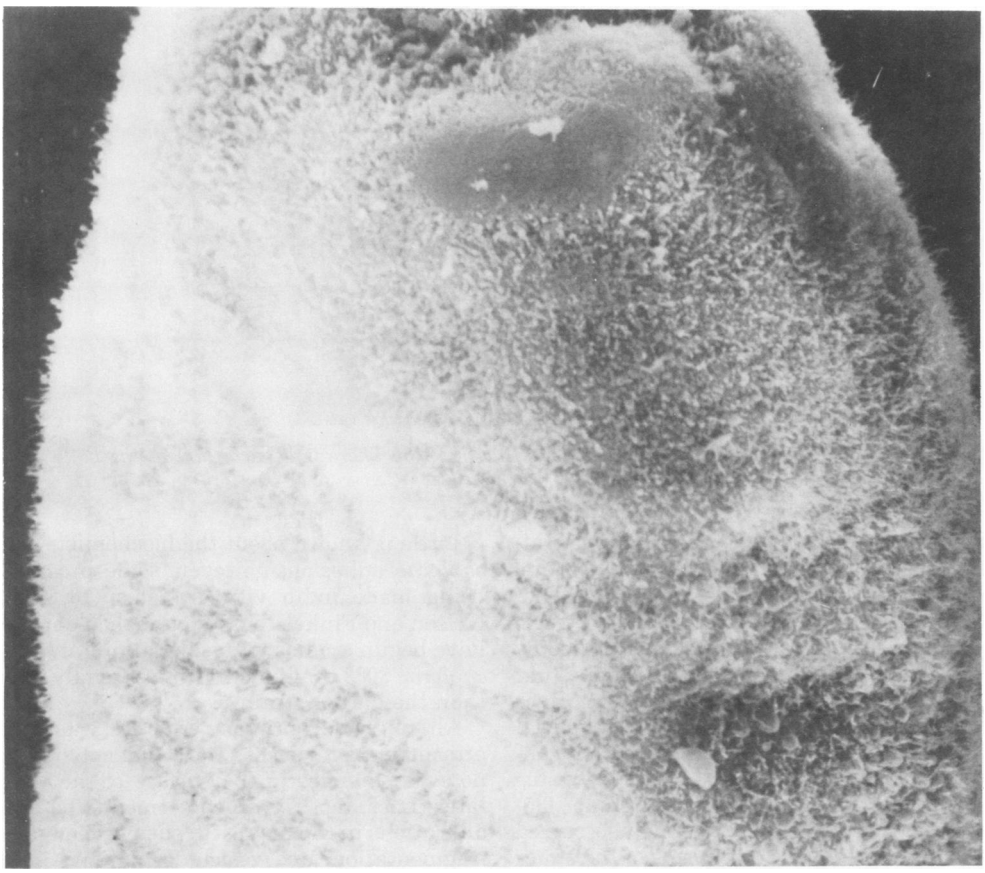

FIG. 14. *Infant rabbit villus showing "fuzzy" appearance due to elongated microvilli 12 h postinfection.* ×*2,160.*

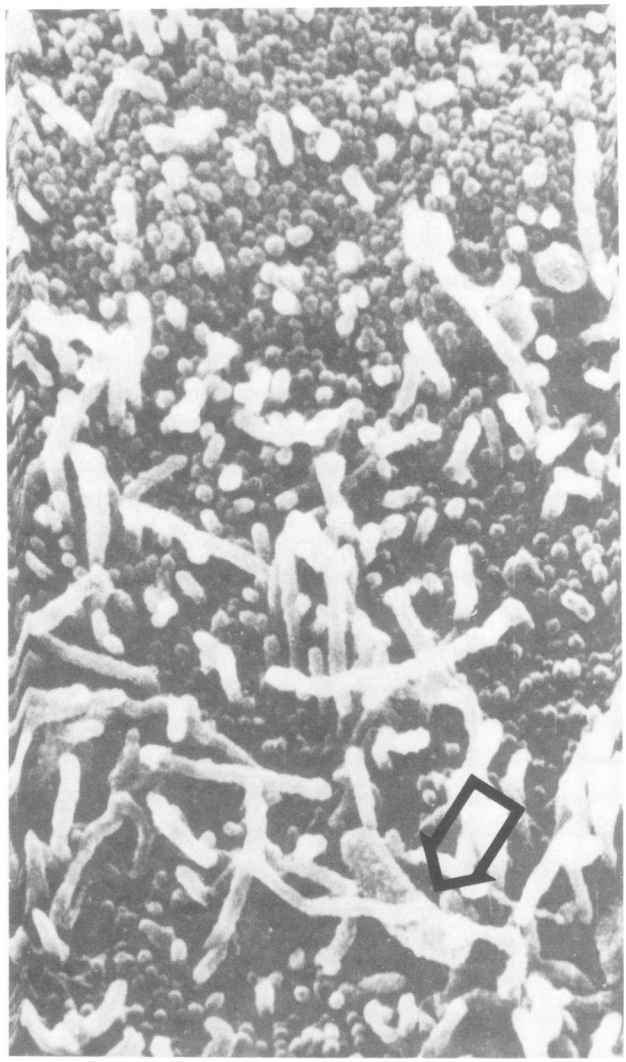

FIG. 15. *Elongated microvilli of infant rabbit villus at 12 h. There is a bacterium at the bottom of the picture (arrow).* ×20,000.

cially at the early times, were observed attached in an end-on manner. Most of these (and the other vibrios also) had their flagella extending out into the lumen. Since the cholera vibrio is a highly motile organism and is propelled by a single polar flagellum, it would be likely that the first contact with the intestinal surface would be front-end contact. After the initial binding, the vibrio may assume a more horizontal position to allow for greater surface-to-surface contact. Savage and Blumershine (35) found that both types of attachment occurred with the autochthonous organisms of the stomach and intestine, although some forms were only attached end-on.

Little is known about the biochemistry of *V. cholerae* adhesion; however, some progress is being made in in vitro systems (16, 23, 29; Nelson and Finkelstein, unpublished data). We have begun studies with several mutants of *V. cholerae* 3083 that bind much less avidly in vivo than the parent strain.

Although our primary interest was in the attachment of vibrios, we could not help but note the changes in the surface of the villi at later times, most of which seemed to be due to alterations in the microvilli such as elongation, fragmentation, and vesicle formation. Similar changes in the brush border microvilli had been noted in an electron microscopic study of the

small intestine in human cholera by Chen et al. (3). These authors suggested that the ultrastructural changes in the microvilli might be related to the abnormal fluid secretion of the gut during cholera.

While this study was in progress, a scanning study of the porcine intestine during infection with enteropathogenic *E. coli*, by Hohmann and Wilson (20), reported that *E. coli* adherent to the epithelial surface were found mainly in the posterior small intestine, whereas *V. cholerae* in our studies colonized the surfaces of the entire small bowel equally well. It is difficult to compare our study with theirs, however, since

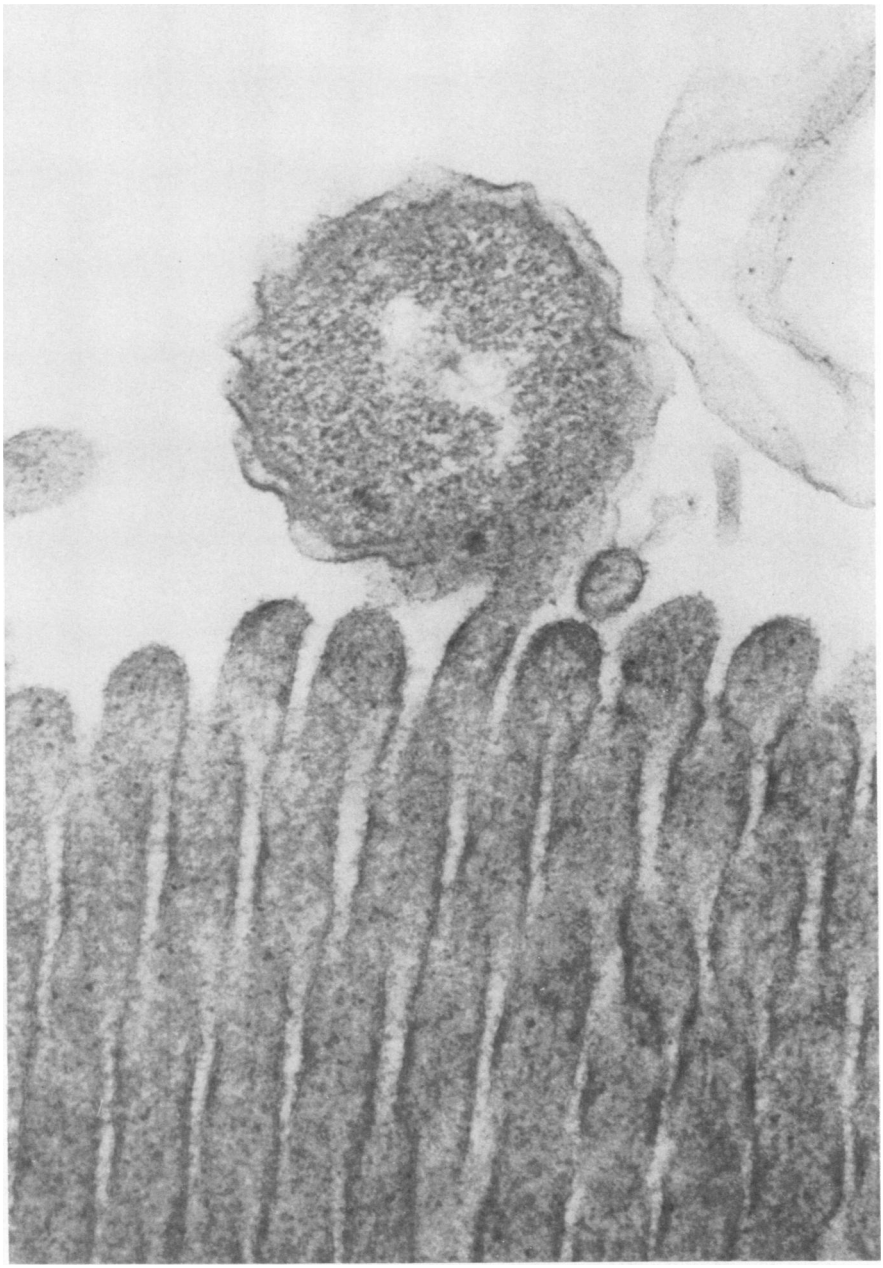

FIG. 16. *Cross section of V. cholerae adhering directly to the edge of the brush border. The outer membrane of the vibrio is in contact with the surface of the microvilli.* ×130,000.

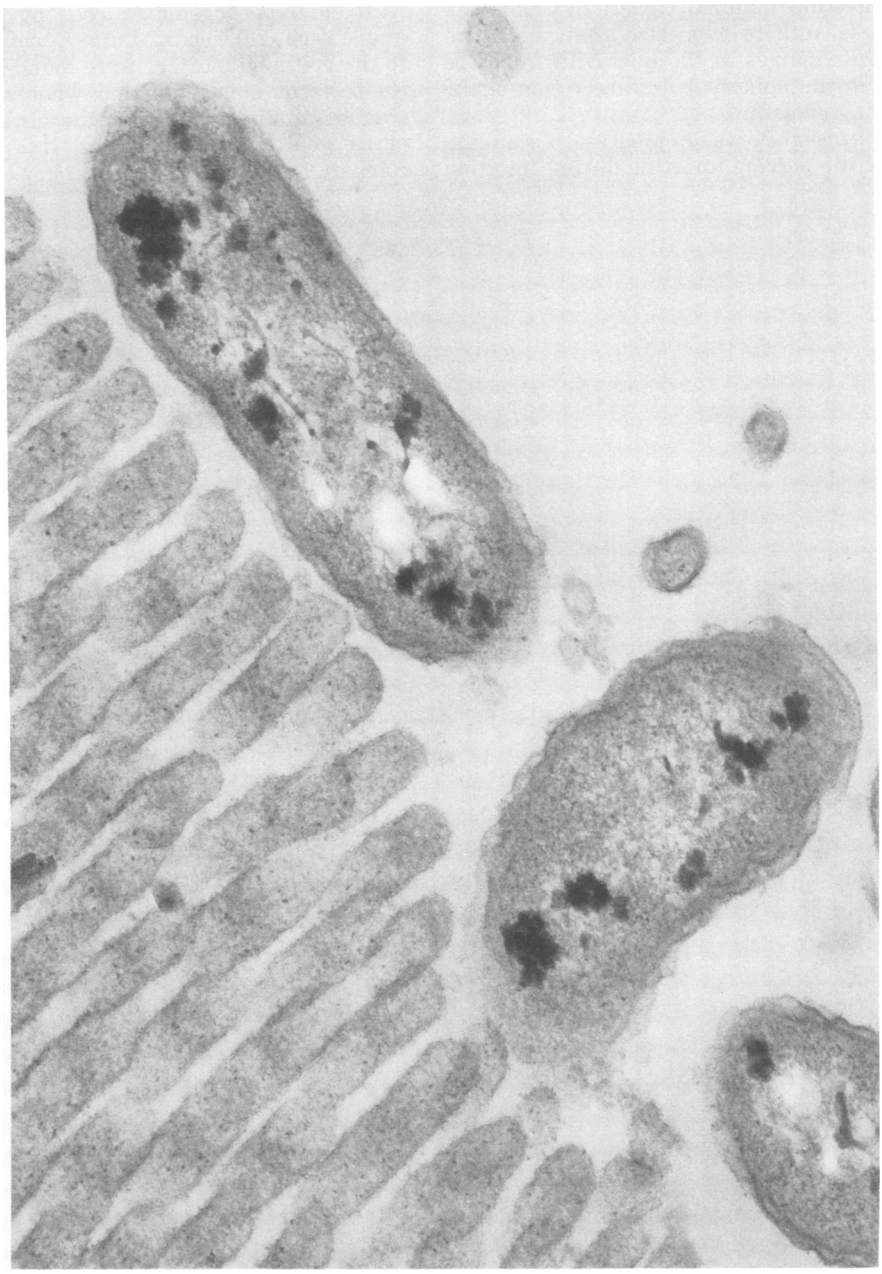

FIG. 17. *Micrograph of V. cholerae adhering to the brush border of a rabbit villus.* ×90,000.

they only looked at one time period (12 to 14 h) after infection. A K88-possessing strain of *E. coli* was found to be separated from the microvilli by an electron-lucent halo, whereas a non-K88-possessing strain attached more like our cholera vibrios. The *E. coli* attached either in patches or more evenly over the villi, depending on the location in the intestine.

Fimbriae (pili) are thought to have a role in the attachment of some bacteria. Negative staining and shadow-casting electron microscopic studies on *V. cholerae* 3083 occasionally showed one or two long, flexible, pili-like structures per cell, and these were not present on all the cells of the culture. Some strains of *V. cholerae*, which had more numerous fimbria-

like structures, were found to be less adhesive both in vivo and in vitro (Nelson, unpublished data). Some vibrio strains also produce a slime layer which might confer adhesiveness (29). Therefore, whereas there is no evidence that pili or other surface structures other than the cell envelope of the strain we have studied play a role in attachment to the intestinal surface, additional study with other strains is needed.

Ultimately, it must be established that simi-

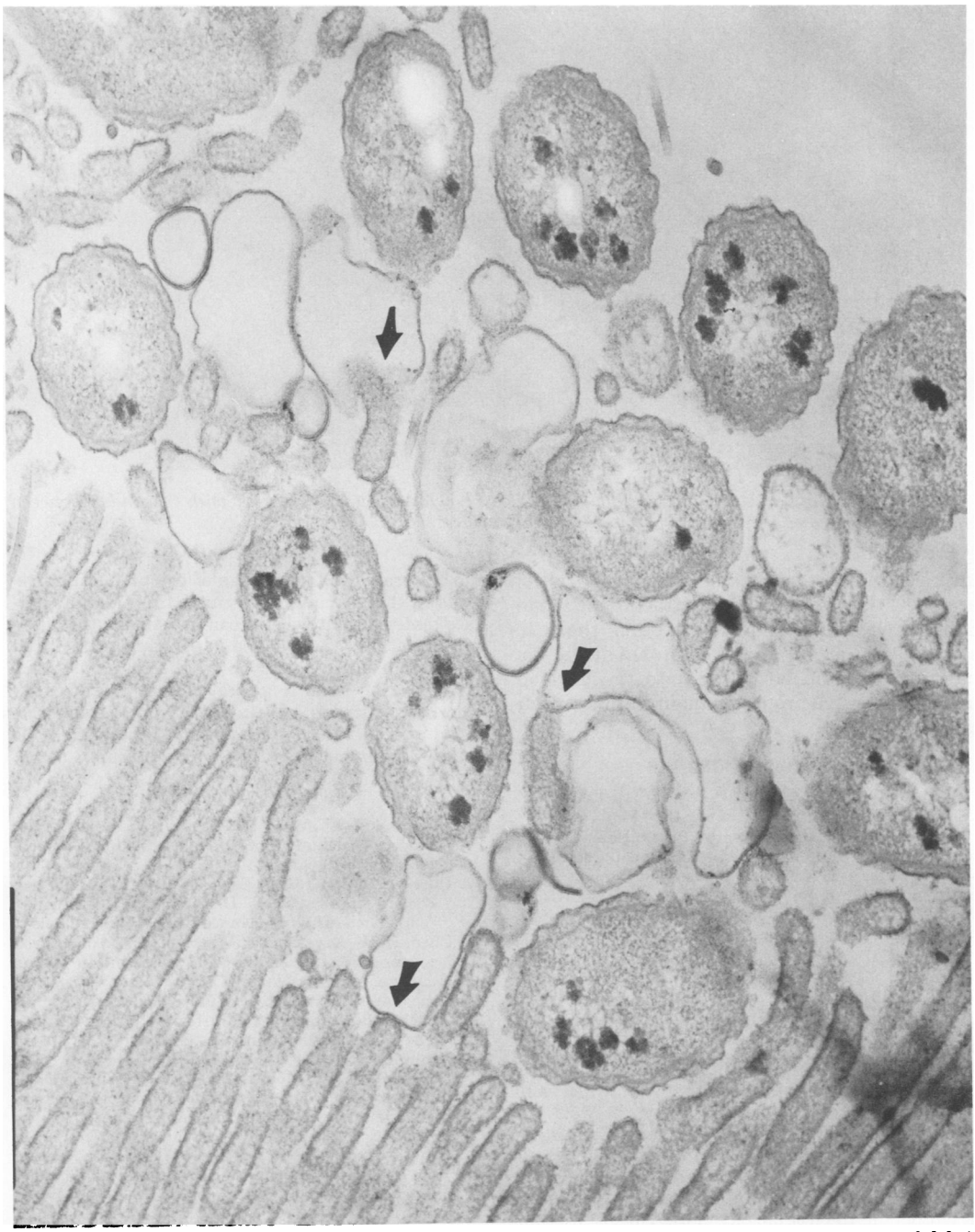

FIG. 18. *Micrograph showing cross section through a group of bacteria. Note the large amount of debris consisting of empty membrane-bound vesicles (arrows) and fragments of microvilli.* ×66,000.

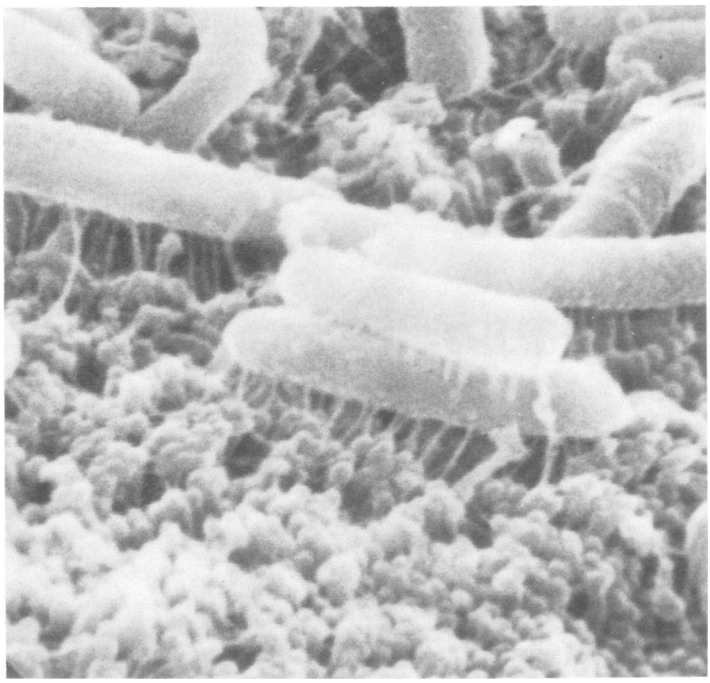

FIG. 19. *Scanning electron micrograph (×27,000) showing groups of vibrios with "strands" between vibrio and vibrio and between vibrio and intestinal surface.*

lar mechanisms of attachment and colonization are operative in humans. If attachment is mediated by surface components other than the somatic antigen or the enterotoxin itself, the mechanism could be relevant to the development of effective prophylactic immunity in humans.

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ADDENDUM

In our continuing effort to understand the mechanism of attachment of cholera vibrios to the intestinal epithelium, we have encountered occasional scanning electron microscopic fields, such as the extreme example provided by Fig. 19, in which well-defined strands of material are evident between the vibrios and the epithelial surface and between vibrios attached to other vibrios. In each of the preparations in which this is observed, the strands are only visible at the zones of attachment at the bottom of, but not on, the free surface of the vibrios. It is our impression, based on the rarity of the observation and the distribution of the material, that it may represent an artifact of the preparation. Perhaps during processing shrinkage of the preparation results in the formation of strands from a slimy surface material, but we still cannot exclude the possibility that pili are involved in attachment and that they are usually destroyed by the manipulations. However, no known alterations in methodology were introduced in obtaining the photograph provided as Fig. 19, in which the vibrios appear to be somewhat further from the microvilli than is usual.

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