Ultrastructural Changes in the Upper Small Intestinal Mucosa in Patients With Cholera

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Background & Aims: Small intestinal mucosal ultrastructural abnormalities were reported in a limited number of patients with cholera in the 1970s. This study extends these observations by examining distal duodenal biopsy samples from 19 patients with cholera and 10 controls. Methods: Endoscopic biopsy samples obtained, usually during the first 24 hours of illness, were processed for electron microscopy. Results: Widening of intercellular spaces and alteration of apical junctional complexes were prominent in the villus epithelium, whereas blebbing of microvillus border and mitochondrial changes were more prominent in the crypt epithelium. The apical junctional and intercellular space changes were not altered by oral rehydration. Degranulation of argentaffin cells, mucosal mast cells, and eosinophils; increase in neutrophil polymorphs; and changes in the enteric nerve fibers and microvasculature were also present. The extent of the changes correlated with clinical severity. Conclusions: The differential involvement of the villus and crypt suggests that factors responsible for secretion may act differentially on surface and crypt epithelium and that both regions may contribute to secretion. The contribution of the enteric nervous system, vasculature, argentaffin cells, mucosal mast cells, eosinophils, and neutrophils in the secretory process and in determining the severity of the clinical illness must be determined by further clinical studies.

Watery diarrhea, especially cholera, is still a major cause of morbidity and mortality in many tropical and subtropical regions. The recent demonstration of organisms other than Vibrio cholerae serotype 01, which can elaborate cholera toxin,1 suggests that this major public health problem will continue to be a threat with potential to develop into pandemics. Induction of secretion in the gut by cholera toxin is now accepted as the basic pathogenic process,2 although it has been suggested that other toxins elaborated by V. cholerae, Ca2+, the enteric nervous system, and vasculature may also play a role.3 As early as 1960,4 it was established that the intestinal epithelium was apparently intact in patients with cholera, a finding confirmed by other light microscopic studies from the Philippines, Thailand, and Italy.5–8 However, the limited reports of ultrastructural changes in mucosal biopsy specimens from patients with cholera9,10 showed significant abnormalities in enterocytes, which seemed to be more than the mere biochemical effect of an enterotoxin. It was suggested that some of the findings reported by these investigators may be related to processing artifacts.11 A detailed reevaluation of the fine structure of the gut epithelium and lamina propria in mucosal biopsy specimens from 19 untreated patients with cholera reported in this article confirms and extends the earlier observations and helps to further the understanding of the pathophysiology of this disease.

Materials and Methods

This study evaluated 19 adults with untreated acute watery diarrhea of <48 hours’ duration (16 were <24 hours), from whom V. cholerae 01 was cultured. Seven patients had moderate and 12 had severe illness as per criteria established earlier by us.12 All patients were initially seen at the hospital emergency services; if they had hanging drop preparations positive for motile curved rods, they were transferred to a metabolic ward. After stabilization with intravenous rehydration, usually within 4–6 hours of admission, 2–3 mucosal biopsy samples were obtained from the junction of the second and third part of the duodenum or the proximal third part through a forward viewing Olympus fiber-optic upper gastrointestinal endoscope (Tokyo, Japan). Four of the patients underwent repeat biopsies 4 hours after they were started on the World Health Organization oral rehydration fluid. All biopsy samples were immediately retrieved, fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and embedded in araldite. Ultrathin sections were cut on an LKB UM4 ultramicrotome (Bromma, Sweden) with a Diamond knife (Diatome, Switzerland), stained with uranyl acetate and lead citrate, and examined with a Philips EM201C electron microscope (Eindhoven, The Netherlands). Biopsy samples were also obtained and processed from similar sites from 10 healthy volunteers. The study was ethically approved by the Institutional Research Committee.

The final analysis was performed on coded sections with the
pathologist unaware of the clinical diagnosis. From each patient and control, 2–4 blocks with well-oriented villi in their full extent with no evidence of crush artifacts were studied. The total villus height and the length of the villus epithelium with dilated intercellular spaces were measured in at least five well-oriented toluidine blue–stained 1-μm-thick sections using a graticule in a light microscope. The length of villi with widened intercellular space was expressed as a percent of total villus length. The cellular infiltrate in the lamina propria was quantitated by counting neutrophil polymorphs, eosinophils, and mucosal mast cells in at least 10 nonoverlapping fields under the oil immersion lens in each patient. The average number of each type of cell per field was calculated in each patient and control. In well-oriented electron micrographs of the apical area of villus enterocytes, the width of the space apparent in the zonula adherens region of the apical junctional complex was measured in at least five cell junctions, and the average width was calculated for each patient. The length of microvilli was taken as the average of five measurements on well-oriented electron micrographs. The Student’s t test was used for testing the significance of group means.

**Results**

**Volunteer Controls**

The appearance of the biopsy samples from volunteer controls were consistent with the changes of tropical enteropathy and similar to findings in biopsy samples from the first loop of jejunum of controls reported earlier. Briefly, there was an increase in the thickness of the crypt layer, increase in lysosomes in villus cells, and scattered dark staining cells with degeneration of cellular organelles. In the lamina propria, scattered venules showed reduplication of basal lamellae, suggesting prior damage. Tight junctions, microvilli, glycocalyx, and intercellular spaces were normal. There was minimal increase in the cellularity of the lamina propria.

**Patients With Cholera**

**Villus epithelium.** Marked widening of the lateral intercellular spaces, usually filled with fluid, was a striking feature in the biopsy specimens taken from all patients (Figure 1). This widening was present only in the upper third to half of the villi and was maximal at the villus tips, gradually decreasing towards the middle of the villus. The basement membrane was intact, and the cell bases were apposed to each other with interdigitating lateral elongations of the basolateral cell membrane (Figure 2). In a few areas toward the tips of the villi, where the widening of the intercellular spaces was most marked, the lateral borders of the cells had lost their interdigitations, the bases of the enterocytes appeared discontinuous, and the adjacent basal lamella was less distinct. Widening of the intercellular space affected 20%–50% of the villus length. The length of villi with intercellular space widening was increased in patients with severe clinical illness (38%) compared with moderately sick patients (34%).

Abnormalities of the apical intercellular junctional complexes were a consistent feature in all patients. The zonula occludens was distorted and often S-shaped, but the membranes of the two adjacent cells were not separated. The immediately subjacent zonula adherens was invariably widened in the upper one third of the villi,
and perijunctional actin condensation around this region was quite marked. The cell membranes were separated in a linear fashion or less frequently as one or more saccules, and the width of this space ranged from 20 to 80 nm. Some of the saccular areas were considerably wider, up to 200 nm (Figure 3A–C). The desmosome adjacent to the zonula adherens was normal, and this enabled easy detection of the widening of the zonula adherens because normally the intermembranous gap in both these areas are similar. This abnormality was present in almost all cells in the upper villi in the area in which the intercellular space was widened. The junctional complexes of enterocytes in the lower half of the villi and the crypts were normal (Figure 3D and E).

The biopsy samples obtained about 4 hours after initiation of oral rehydration fluids were carefully examined to see whether the widening of the lateral intercellular space and the junctional complexes showed any changes due to fluid absorption. The appearances of these biopsy specimens were identical to the initial biopsy samples from the same patients. Specifically, there was no alteration in the zonula occludens area.

Microvilli were intact but shorter than in controls (controls, 1.02 ± 0.2 m; patients, 0.83 ± 0.17 m). There were scattered areas of actin condensation surrounding microvillus rootlets. Irregular blebbing of the microvillus border was a consistent finding in about 5%—8% of the enterocytes in the upper one third of villi in all patients. The mitochondrial matrix was dense with a few small, round, well-defined electron-dense deposits in all biopsy samples (Figure 4). Electron-dense bodies and lysosomes were increased in villus enterocytes. In biopsy specimens from 7 patients, there were focal infiltrations of the epithelium by single or groups of neutrophil polymorphs (Figure 5). Small droplets of fat were present in the supranuclear and infranuclear cytoplasm of enterocytes in 7 patients. In intervillus areas, bacterial bodies resembling V. cholerae, enmeshed in extensions of the glyocalyx and close to microvilli, were present in 3 patients, all of whom underwent biopsy within the first 24 hours of illness (Figure 6).

Crypt epithelium. Widening of the lateral intercellular spaces was strikingly less marked in the crypt epithelium and found only in 10 of the patients, affecting less than one fourth of the cells in the crypts. The zonula

Figure 3. Apical junctional complex of enterocytes from (A) a control and (B–E) patients with cholera (original magnification 27,300×). (A) Control upper third of villus. Note tightly apposed cell membranes in zonula occludens region (arrow), widened space between cell membranes in zonula adherens (arrowhead), and the subjacent desmosome. (B) Upper third of villus of a patient with cholera. Note distortion of zonula occludens and widening of zonula adherens with surrounding actin condensation. Desmosome is intact. (C) Saccular dilatation of zonula adherens upper third of villus. (D) Lower third of villus from biopsy sample shown in B. Relatively normal appearing junctional complex. (E) Crypt from biopsy sample shown in B with normal junctional complex.

Figure 4. Portion of enterocyte from the upper third of the villus from a patient with cholera showing dense, small, round deposits in mitochondria (original magnification 12,500×).
occludens, zonula adherens, and desmosomes of the apical junctional complexes of the crypt enterocytes were normal in appearance without widening or distortion (Figure 3E). However, blebbing of the microvillus surface was more prevalent compared with the villus enterocytes affecting almost half of the crypt enterocytes (Figure 7). In 15 patients, the crypt lumen was widened and contained empty membrane-bound vesicles. Small, round, electron-dense bodies in the mitochondria were larger than in the villus epithelium. The electron-dense bodies were present only in the mitochondria of enterocytes and were consistently not found in Paneth cells or other cells in the epithelial layer. Degranulation of argentaffin cells with vacuolization of the cytoplasm was noticed in 14 patients, usually with adjacent nonmyelinated nerve fibers or mucosal mast cells (Figure 8). Infiltration of the crypt epithelium with single or groups of neutrophil polymorphs were found in 7 patients (Figure 9).
Lamina propria. The mean neutrophil polymorph and eosinophil counts per field in patients with cholera were significantly higher than in controls (Figure 10). This was associated with leukocytosis in the patients (Table 1). The mucosal mast cells were increased in some patients. Degranulation of mucosal mast cells was a striking feature in all patients, particularly when they were near nonmyelinated nerve fibers (Figure 11A), small blood vessels (Figure 11B), and eosinophils (Figure 11C).

Exocytosis of eosinophil granules occurred either in pericellular spaces (Figure 11C) or in degranulation chambers (Figure 11D). Axonal necrosis with swelling and loss of organelles was present in a few of the nonmyelinated nerves in all patients. There was an apparent increase in plasma cells in all patients with plasmacytolsis of pericapillary plasma cells.

The lamina propria blood vessels were markedly congested and dilated, especially in the upper villi in all patients, and there were areas of hemorrhage in the lamina propria. The lumen of the blood vessels contained fibrin strands, increased platelets, platelet thrombi (Figure 12A), and red blood cells with rouleaux formation, distortion, fragmentation, and dehemoglobinization. Capillary and venular endothelium was swollen with rarefaction of the cytoplasm and dilatation of the rough endoplasmic reticulum with platelet and neutrophil polymorph adhesion. Other features of endothelial damage included mitochondrial swelling with loss of cristae, prominent microvillus formation, and intracytoplasmic stress fibers (Figure 12B). In addition to these degenerative changes, there were other endothelial cells showing regenerative activity with prominent Golgi and increase in Weibel–Palade bodies. Interrendothelial cell junctions were normal but, in markedly dilated vessels, there was separation of the junctions with platelet adhesion (Figure 12C). There was an increase in microinocytic vesicles in the cytoplasm of capillary endothelial cells and pericapillary edema (Figure 12C). The capillary pores were intact, although some were enlarged and occasionally contained insinuated red blood cells and fibrin. In some

Table 1. Comparison of Morphological Features Between Patients With Cholera With Moderate and Severe Clinical Illness

<table>
<thead>
<tr>
<th>Severity of Illness</th>
<th>Moderate (n = 7)</th>
<th>Severe (n = 12)</th>
</tr>
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<tbody>
<tr>
<td>% Villus length with widened intercellular space</td>
<td>44</td>
<td>38</td>
</tr>
<tr>
<td>Microvillus length (m)</td>
<td>0.83</td>
<td>0.75</td>
</tr>
<tr>
<td>Width of zonula adherence (nm)</td>
<td>40.00</td>
<td>55.00</td>
</tr>
<tr>
<td>Neutrophil polymorphs per field</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Mucosal mast cells per field</td>
<td>2.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Eosinophils per field</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>White cell count/mm³</td>
<td>7500</td>
<td>13,300</td>
</tr>
<tr>
<td>Plasma bicarbonate (mEq/L)</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>Serum creatinine (g/L)</td>
<td>1.16</td>
<td>1.68</td>
</tr>
<tr>
<td>Serum protein (g/L)</td>
<td>69</td>
<td>91</td>
</tr>
<tr>
<td>Mean systolic blood pressure (mm Hg)</td>
<td>107</td>
<td>88</td>
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NOTE. All parameters except neutrophil polymorphs were worse in patients with severe illness, but none of them achieved statistical significance.
Figure 11. (A) Mucosal mast cell encircling an enteric nerve (original magnification 8300×). (B) Mucosal mast cell adjacent to a capillary. Endothelial cell E1 shows rarefaction of cytoplasm and E2 shows irregular cytoplasmic projections into the lumen (original magnification 8600×). (C) Eosinophil (E) with extruded granular material (arrow). Mucosal mast cell (M) surrounds the eosinophil (original magnification 15,600×). (D) Eosinophil with partial loss of crystalline cores in some granules (arrow) and cytoplasmic degranulation chamber (D) (original magnification 10,400×).

areas, there was dehiscence of capillaries and venules with frank hemorrhage into the lamina propria (Figure 12D).

**Discussion**

The findings reported within this article confirm and extend the earlier reports⁹,¹⁰ that, although the small intestinal mucosa in patients with cholera seems histologically intact, there are significant ultrastructural abnormalities in the villus and crypt enterocytes and the lamina propria. The earlier reports described widening of the lateral intercellular spaces, projections of the microvillus border, swelling of mitochondria, increased Golgi vesicles, and dilatation of the rough endoplasmic reticulum. Apical junctional complex abnormalities with electron-dense bodies in mitochondria; neutrophil polymorph infiltration of the epithelium and lamina propria; degranulation of argentaffin cells, mast cells, and eosinophils; and vascular changes have not been previously reported. The absence of these changes in biopsy samples from controls processed identically clearly establishes that the observed abnormalities are not artifacts of processing. All of the measured parameters of morphological alterations and clinical indicators, such as the total white cell count, the serum creatinine concentration, and plasma proteins, were more abnormal in the 12 patients with severe clinical illness compared with the patients with moderate illness (Table 1).

The apical junctional changes consisted of distorted, but not widened, zonula occludens and widened zonula adherens with perijunctional actin condensation and morphologically normal desmosomes. These changes were marked in the villus epithelium with widened lateral intercellular space and were more marked in patients with severe clinical illness (Table 1). The crypts had
normal apical junctional complexes. The earlier reports\textsuperscript{9,10} did not mention abnormalities of the tight junctions, possibly because 4 of their 6 patients underwent biopsies on the third day of illness or later when the secretory process was likely to be less active, whereas in this study, all biopsy samples were obtained within 48 hours and the majority within 24 hours of onset, while the patient was still having large-volume diarrhea.

The pathogenesis of these apical junctional changes is intriguing. In experimental animals, widening of the zonula occludens with reduction in the zonula occludens strands and actin condensation has been shown to occur in sodium-coupled solute and water absorption.\textsuperscript{14,15} Experimental evidence from studies in other epithelia suggests that increase in cyclic adenosine monophosphate levels may also affect tight junctions.\textsuperscript{16} It has also been shown in experimental studies that villus enterocytes may also be able to contribute to intestinal fluid secre-
The widening of zonula adherens and lateral intercellular spaces in all of the untreated patients, their association with more severe clinical illness, and the absence of any change in the appearance when active absorption was induced by a glucose containing solution suggests that the abnormalities in our patients are likely to be the morphological counterpart of intestinal secretion. A second enterotoxin (zonula occludens toxin) elaborated by \textit{V. cholerae} has been shown to affect the tight junctions in the rabbit ileal loop model,\textsuperscript{18} causing increased permeability, increased tissue conductance and ultrastructural changes characterized by perijunctional actin condensation, increased permeability to luminal horseradish peroxidase in transmission electron microscopy, and decreased zonula occludens strands by freeze fracture studies. Further work is needed to confirm whether zonula occludens toxin plays a role in the pathogenesis of the apical junctional changes in patients with cholera.

The electron-dense deposits in the mitochondria resemble the dense matrix granules described in cardiac muscle cells. It has been suggested that they contain Ca\textsuperscript{2+} in addition to complex proteins and are a reflection of an altered metabolic state.\textsuperscript{19} It is difficult to decide whether these granules are the result of an increase in the Ca\textsuperscript{2+} concentration during the secretory response to cholera toxin or whether it is just a reflection of enhanced metabolic activity in the secreting cell. The differential distribution of these granules, more in the crypt enterocytes, which also have increased blebbing of the microvillus membrane, and less in villus cells, which have dilated paracellular spaces and altered apical junctional complexes, raises questions regarding the differential effects of the factors responsible for secretion on the crypt and villus epithelium. It is generally accepted that the site of fluid secretion in cholera is the crypt region and, in the surface enterocytes, the sodium-solute cotransport system is intact.\textsuperscript{20} The presence of abnormalities in both the crypt and the villus enterocytes and the failure of oral rehydration fluids to change the apical junctional abnormalities in the villus epithelium, suggest that both these regions are involved in the disease process. The alteration in the apical junctional complex with widening of zonula adherens is different from the changes induced by sodium-solute cotransport in which the widening occurs in the zonula occludens.

The lamina propria vasculature was markedly congested and damaged with neutrophil polymorph and platelet adhesion, intravascular fibrin strands with endothelial cell damage, and dehiscence. Studies on experimental cholera in rabbit ileal loops\textsuperscript{21–24} had earlier shown dilatation of capillaries and increased escape of intravenously injected horseradish peroxidase from the vessels. The toxin preparation used in these studies was broth filtrates, which are likely to have contained other metabolites and endotoxin in addition to cholera toxin. Earlier we reported\textsuperscript{25} similar vascular changes in the rectal mucosal lamina propria in adults with acute diarrhea including cholera, which correlated with the clinical severity of the illness. The vascular changes resemble the abnormalities in the local Schwartzman reaction triggered by gram-negative bacterial endotoxin. We have also described a murine model of acute watery diarrhea in response to parenteral endotoxin challenge.\textsuperscript{26} If bacterial endotoxin does play a role in determining the severity of the clinical illness by contributing to vascular damage, it could be possible to mitigate clinical severity responsible for morbidity and mortality by blocking this action. Experiments with purified cholera toxin are needed to determine if it can also directly affect blood vessels and result in an increase in neutrophil polymorphs.

Cholera is considered to be the classical paradigm of noninflammatory toxigenic diarrhea. Earlier studies have noted neutrophil polymorphs in the lamina propria in patients with cholera\textsuperscript{6} and in some animal models.\textsuperscript{23} Reports from Bangladesh of elevated acute phase reactant proteins and leukocytes in the blood\textsuperscript{27} and increased neutrophil polymorphs in cholera stool\textsuperscript{28} are supported by the present observations of increased neutrophil polymorph infiltration of the epithelium and the lamina propria. Striking alterations in argentaffin cells, mast cells, eosinophils, and enteric nerves reported in these biopsy samples from patients suggest that there are likely to be other major factors that contribute to the pathogenesis of fluid secretion in cholera. Experimental studies have suggested roles for argentaffin cells, 5-hydroxytryptamine, and the enteric nervous system in the secretion in cholera.\textsuperscript{29–32} The complex interaction of mast cells, eosinophils, and enteric nerves\textsuperscript{33} and the critical role of the interaction of intestinal mediators in cholera\textsuperscript{34} are now being recognized. The abnormalities in all of these cell types found in patients with cholera in this study suggest that a new paradigm for infectious diarrhea suggested recently\textsuperscript{35} is likely to be of crucial importance. Further studies on clinical material are likely to help us to understand the complex pathophysiology of secretion in cholera and other watery diarrhea and refine our approaches to management.

\textbf{References}


