

Acute Infectious Nonbacterial Gastroenteritis: Intestinal Histopathology

Histologic and Enzymatic Alterations During Illness Produced by the Norwalk Agent in Man

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Intestinal biopsy specimens were obtained from normal volunteers before, during, and after administration of the Norwalk agent of acute infectious nonbacterial gastroenteritis. The small intestine of acutely ill volunteers had an intact mucosa but showed histologic changes that included blunting of villi, shortening of microvilli, dilation of endoplasmic reticulum, and an increase in intracellular multivesicular bodies. Convalescence biopsy specimens, obtained 2 weeks after illness, showed normal histologic patterns. Specimens obtained from volunteers who remained clinically well did not show histologic changes at any time. At the time of illness, brush-border enzyme activities (alkaline phosphatase, sucrase, and trehalase) were decreased as compared with base-line and convalescent values. Thus definite but reversible pathophysiologic lesions of the small intestine in man were seen during acute gastroenteritis induced by a viral agent.

ACUTE INFECTIOUS nonbacterial gastroenteritis is a self-limited disease characterized by diarrhea, vomiting, abdominal cramps, and malaise (1). The disease often occurs in explosive epidemic outbreaks, lasting 24 to 48 hours and remitting spontaneously without sequelae (2). The general term acute infec-

tious nonbacterial gastroenteritis includes a variety of syndromes, such as viral diarrhea (3), winter vomiting disease (4), epidemic nausea and vomiting (5), and epidemic collapse (6). Although this type of disease is extremely common, ranking second only to upper respiratory tract infections as a source of morbidity in the United States (7), definitive cultivation of causative agents in vitro has not been possible (8). In studies previously reported, biological and physical properties of one such agent, derived from a typical outbreak in Norwalk, Ohio, in 1968 (9), were elucidated for the first time (10, 11). This agent appears to have the properties of a small (27 nm), heat-stable, ether- and acid-resistant virus.

Except for an isolated report (12), evidence linking established viruses with outbreaks of gastroenteritis has been largely inferential. Thus, descriptions of the pathophysiology of the syndrome have been limited to case studies in naturally occurring outbreaks in which known causative agents have not been found and have, therefore, been presumed to be "viral" (13). The histopathology of the above-mentioned clinical syndromes of gastroenteritis caused by a documented viral agent in man has not been previously reported. The recent availability of the Norwalk agent enabled us to investigate this problem by examining morphologic and biochemical alterations in the human intestine in experimentally induced viral gastroenteritis.

Subjects and Methods

CLINICAL STUDIES

Volunteer experiments were conducted at the Clinical

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Center of the National Institutes of Health under close hospital supervision, as previously described (14).

Volunteers consisted of seven men, ages 19 to 21 years, from the normal volunteer program of the NIH.

All volunteers gave informed consent and had unremarkable histories and physical examinations. Hematocrit, leukocyte count, urinalysis, and routine serum chemistries, including liver function studies and serum carotene values, were within normal limits.

INOCULUM

The inoculum containing the Norwalk agent was a diarrheal stool filtrate obtained after two serial passages of the agent in volunteers. It was shown to be free of bacterial, fungal, parasitic, and known viral agents and enterotoxin as described in previous studies (15).

After an overnight fast, volunteers ingested 2 g of NaHCO₃. Five minutes later they drank 5 ml of the clear, odorless filtrate, diluted in 30 ml of water.

CRITERIA FOR ILLNESS

Only volunteers who developed documented vomiting or diarrhea (unformed stool) were considered to have been ill. Volunteers who had symptoms such as malaise, headache, abdominal cramps, and anorexia in the absence of diarrhea or vomiting were not considered to have been ill.

INTESTINAL BIOPSY

Peroral intestinal biopsies were done with a Rubin tube positioned fluoroscopically at the level of the ligament of Treitz (16). Three to four specimens were generally obtained. Base-line biopsies were obtained before inoculation in six out of the seven volunteers. A second biopsy was performed on all volunteers, either at the height of illness or 48 to 72 hours after inoculation in clinically well volunteers. Convalescence biopsies were obtained 2 weeks after inoculation. Rectal biopsies were obtained at sigmoidoscopy at the time of illness in two volunteers.

Biopsy specimens were fixed in formol-sublimate (B5) fixative and stained with hematoxylin and eosin for light microscopy. Other pieces were fixed in 10% neutral formalin and stained with oil red O for lipid. Specimens for electron microscopy were fixed in cold (4 °C) 1.25% glutaraldehyde solution in phosphate buffer (pH 7.35) immediately after excision. They were postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graduated steps in ethanol, stained *en bloc* with 2% uranyl acetate in ethanol, and embedded in an epon-araldite mixture. Diamond-knife ultrathin sections (approximately 50 to 60 nm thick) were stained with 0.1% lead citrate (pH 12), and electron microscopy was performed with an AEI electron microscope*.

ENZYME ASSAYS

Biopsy specimens were homogenized in ice-cold 0.85% saline, and aliquots were assayed for sucrase, lactase, and trehalase by a modification of the method of Dahlqvist (17). Sucrose, lactose and trehalose† were

prepared in a concentration of 0.056 M in 0.1 M maleate buffer at pH 6.0. One tenth millilitre of intestinal homogenate was incubated with 0.1 ml of substrate at 37 °C for 1 to 4 hours. The reaction was stopped by placing the tubes in a boiling water bath for 2 minutes. Liberated glucose was measured by the glucose oxidase method. Disaccharidase activity was expressed as micromols of glucose per gram protein per minute.

Alkaline phosphatase was assayed according to the method of Bessey, Lowry, and Brock (18). The standard reaction mixture contained 50 µl of diluted homogenate and 200 µl of 0.15 mM *p*-nitrophenyl-phosphate† in 0.0625 M glycine buffer at pH 10.3. The glycine buffer contained magnesium chloride (0.625 mM) and zinc acetate (0.125 mM). After a 10- to 30-minute incubation at 37 °C, the reaction was stopped by the addition of 1.0 ml of 0.02 N NaOH. Homogenate blanks were inactivated with 0.02 N NaOH and then incubated at 37 °C for the same time. Phosphatase activity was expressed as micromols of *p*-nitrophenol liberated per gram of protein per minute. The assay has been shown to be linear up to 30 minutes, and the quantity of substrate liberated is a function of the amount of homogenate added (18). Proteins were measured by the method of Lowry and associates (19).

Results

CLINICAL

Four of the seven volunteers who received the inoculum developed clinical illness as defined above. Three out of the four had diarrhea, two had vomiting, two had low-grade fever (37.7 °C and 38.5 °C, orally), three had abdominal cramps, and three had headache. The incubation period of the illness was 16 to 36 hours, with a duration of 24 to 48 hours. All four volunteers recovered completely without treatment and without sequelae.

The three volunteers who did not develop diarrhea or vomiting remained asymptomatic after inoculation.

HISTOLOGY

Base-line intestinal biopsies performed on six volunteers up to 2 weeks before inoculation showed a normal villous architecture with a normal epithelial lining in five of six cases (Figure 1A). In one case there was villous broadening, but this subject was included in the study after demonstration of normal jejunal epithelial cells by both light and electron microscopy.

The four volunteers who developed clinical illness were biopsied at the height of the disease, 24 to 48 hours after inoculation. Intestinal mucosa in four had light-microscopic abnormalities that included partial villous flattening, broadening of the villi, and disorganization of the epithelial lining cells (Figure 1B). Three of the volunteers had a moderate infiltration of the lamina propria by mononuclear cells, and there

* AEI Scientific Apparatus Inc., Elmsford, New York.

† Sigma Chemical Co., St. Louis, Missouri.

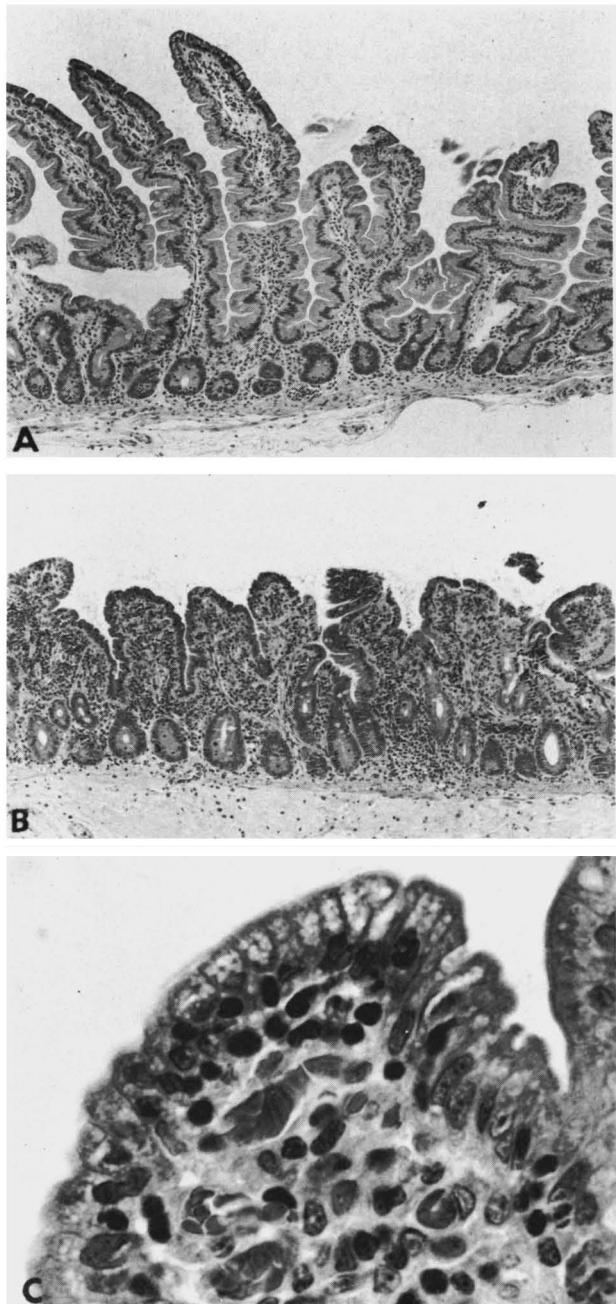


Figure 1A. Light micrograph of intestinal biopsy from Volunteer A taken before administration of inoculum. Villi and cellular morphology appear normal. (Hematoxylin and eosin; magnification, $\times 100$.) **B.** Light micrograph of intestinal biopsy from Volunteer A at the time of illness. Villi are broadened and flattened; epithelial lining cells appear disorganized. (Hematoxylin and eosin; magnification, $\times 100$.) **C.** Light micrograph of intestinal biopsy from Volunteer B at the time of illness. Focal areas of epithelial cell vacuolization by a non-lipid-staining material are seen. (Hematoxylin and eosin; magnification, $\times 1000$, oil immersion.)

were focal areas of epithelial vacuolization by a non-lipid-staining material (Figure 1C).

Electron microscopy showed definite changes in the jejunal epithelial cells of the ill patients. Dilatation of the rough and smooth endoplasmic reticulum

was noted, along with an increase in multivesicular bodies (Figures 2 and 3). In three of the four the microvilli were shortened, and the intercellular spaces were widened and filled with an amorphous electron-dense material (Figure 3). No definite viral particles were noted. Convalescence biopsies done 2 weeks after inoculation showed that the mucosa had returned to normal in all cases (Figure 4).

The three volunteers who did not become clinically ill were biopsied 48 hours after inoculation. In all three the jejunal histology was normal by light microscopy. Electron microscopy showed no changes in these postinoculation specimens as compared with their base-line and convalescence specimens.

Rectal biopsy specimens obtained in two cases at the height of illness were histologically normal.

ENZYMOLOGY

Brush-border enzyme activities were assayed in the base-line, the postinoculation, and the convalescence biopsy specimens. One volunteer who subsequently developed clinical illness did not have a base-line biopsy done. Another volunteer had a normal base-line alkaline phosphatase value, but his di-saccharidase values were not measured.

Since relative changes were the quantities of interest, the enzyme level in the specimen obtained from each volunteer 24 to 48 hours after inoculation was compared with the geometric mean of his own base-line and convalescence levels of that enzyme. The arithmetic means of these ratios, expressed as percent changes, were compared between ill and well groups, using Student's *t* test. Convalescence enzyme levels had returned to the base-line range in all volunteers and were therefore averaged with initial base-line levels in this analysis.

The results are summarized in Table 1. The percent changes of alkaline phosphatase levels in ill volunteers (-49.3%) and well volunteers ($+1.4\%$) were significantly different ($P < 0.01$). Similarly, the percent change in trehalase activity at the time of illness (-61.7%) was significantly different ($P = 0.02$) from that seen in well volunteers (-16.2%). However, when percent changes of sucrase levels were similarly compared, the changes were not statistically significant, even though the sucrase levels were decreased during illness.

Lactase levels of the 48-hour intestinal biopsy specimens of ill volunteers were lower than base-line and convalescence levels. However, because of the wide range of lactase values in both the ill and well groups, the difference between the two was not significant.

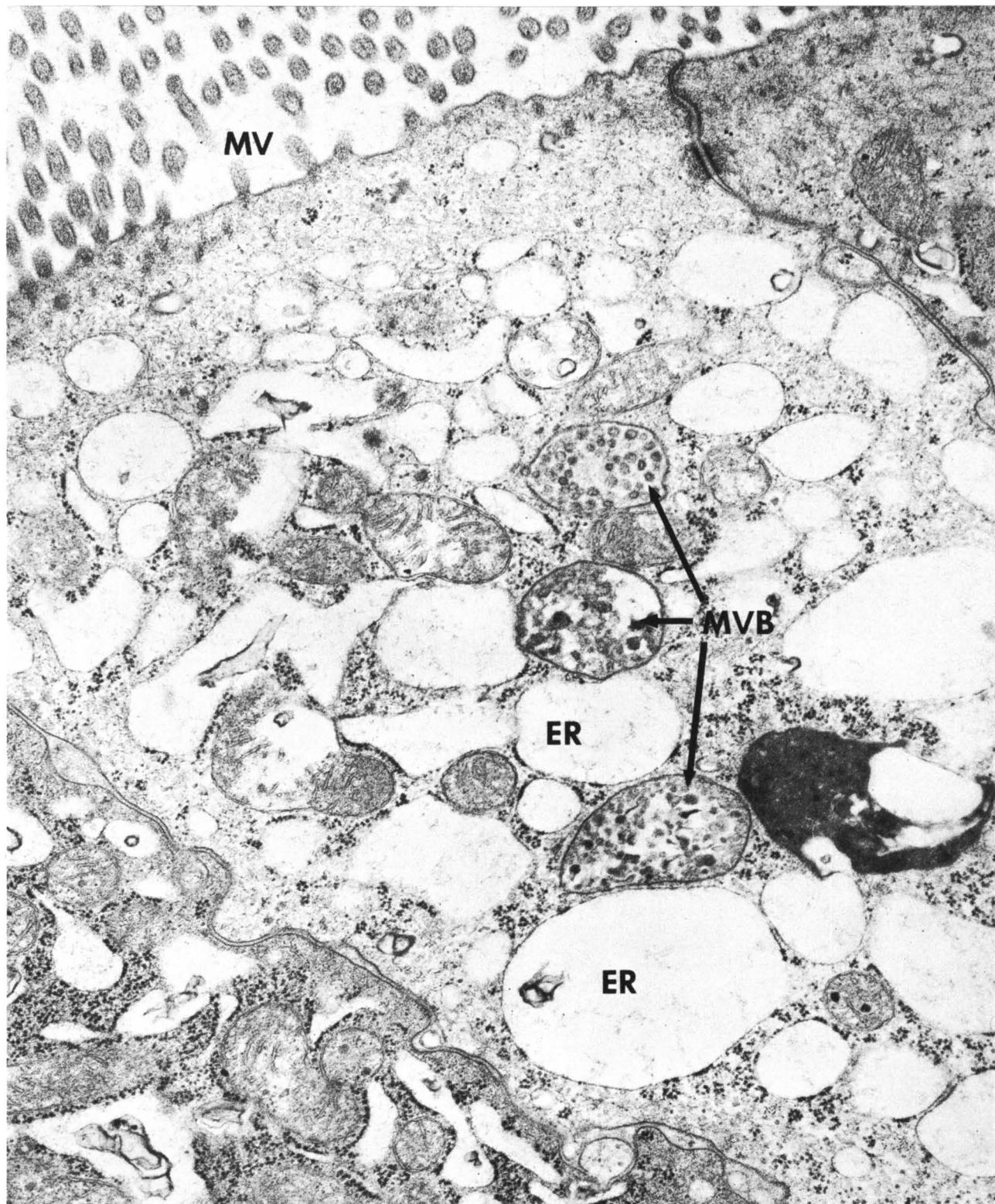


Figure 2. Electron-microscopic appearance of intestinal epithelial cells from mid-villus region in Volunteer A at the time of illness. Prominent changes include dilation of endoplasmic reticulum (ER) and the presence of large numbers of multivesiculate bodies (MVB). Microvilli (MV) are present in tangential and cross section. (Pb-citrate stain; Plate #BRC-18,389; electron-microscopic magnification, $\times 10\,000$; final magnification, $\times 31\,000$.)

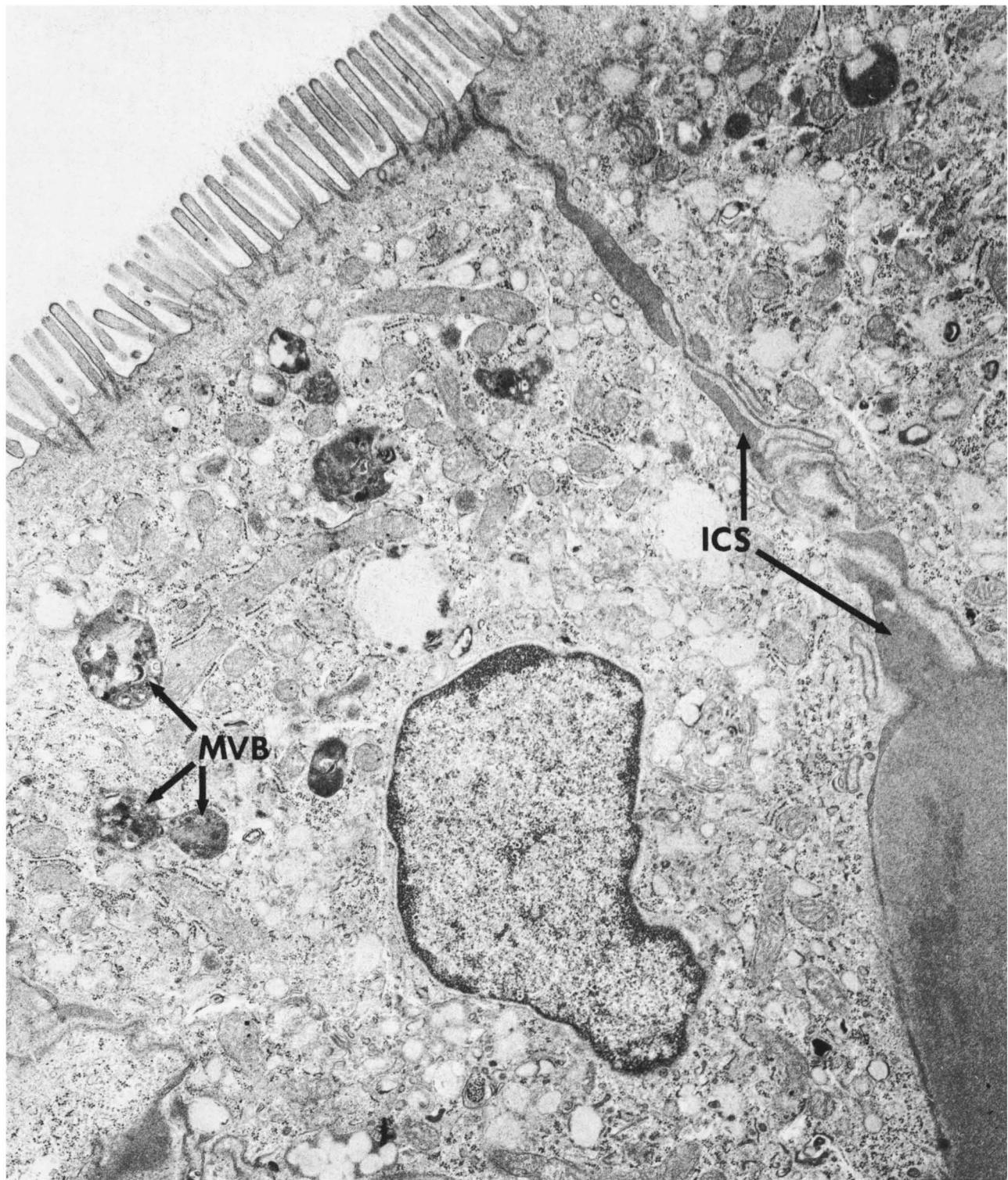


Figure 3. Electron micrograph of intestinal biopsy specimen from mid-villus region of Volunteer B at the time of illness. In addition to changes noted in Figure 2, microvilli are shortened, and intercellular spaces (ICS) are widened and filled with amorphous electron-dense material. (Pb-citrate stain; Plate #BRC-18,341; electron-microscopic magnification \times 5000; final magnification, \times 15 500.) MVB = multivesicular bodies.

Discussion

Descriptions of the pathological findings in viral infections of the gastrointestinal tract have been limited

to those viruses that have primarily extragastrointestinal manifestations or to outbreaks of gastroenteritis in which no causative agent has been found. In varicella infection in man, Sheehy, Artenstein, and Green

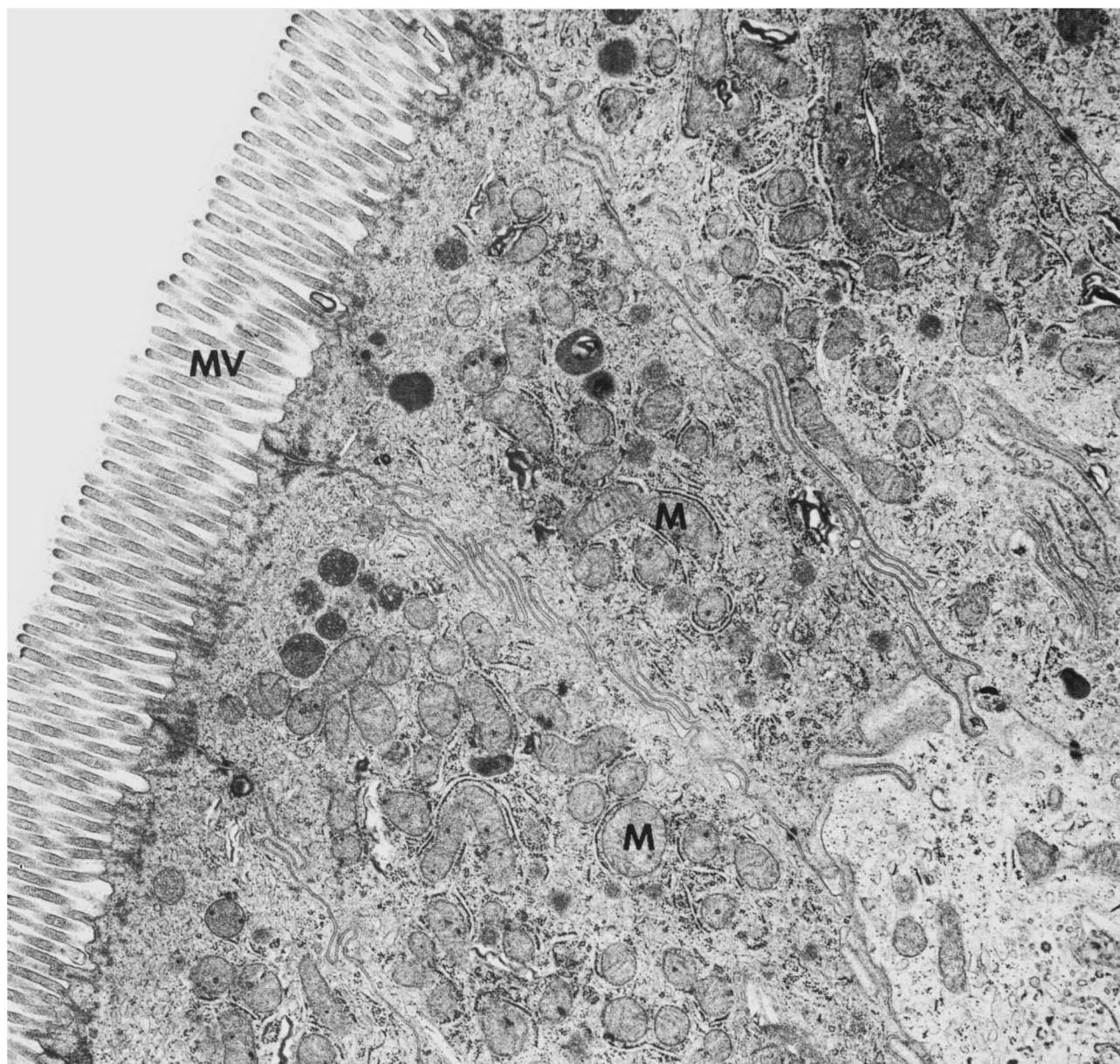


Figure 4. Electron micrograph of convalescence biopsy specimen from Volunteer B, showing normal columnar intestinal epithelial cells with normal microvilli (MV) and abundant mitochondria (M). (Pb-citrate stain; Plate #BRC-71426-1B; electron-microscopic magnification, $\times 5000$; final magnification, $\times 15\,500$.)

(20) found an intact intestinal mucosa with subepithelial edema and mild round cell infiltration in the lamina propria. In infectious mononucleosis (20) the lamina propria was filled with atypical lymphocytes and eosinophils, and vesicles were seen in the cytoplasm of epithelial cells at the tips of the villi. In rubella infection (20) the mucosa appeared to be normal, whereas the lamina propria had mild round cell infiltration. The most marked changes have been found in hepatitis, in which Conrad, Schwartz, and Young (21) have observed severely blunted jejunal villi, with flattened epithelial cells and round cell infiltration of the lamina propria. Sheehy and Floch

(13) performed intestinal biopsies on several patients with a moderate afebrile enteritis of obscure etiology in Puerto Rico. The jejunal mucosa showed extensive flattening and shortening of the villi, necrosis of epithelial cells, and inflammatory infiltration of the lamina propria.

In our studies with the Norwalk agent, jejunal biopsies at the height of illness have shown partial villous flattening, disorganization of the epithelial cells, vacuolization of the cytoplasm, and mononuclear infiltration of the lamina propria. Increased numbers of lysosomal bodies were seen, along with dilation of the endoplasmic reticulum and loss of

Table 1. Intestinal Enzyme Activities in Sequential Biopsy Specimens from Volunteers Inoculated with Norwalk Agent of Acute Infectious Nonbacterial Gastroenteritis

Intestinal Enzyme	Volunteer Group	Activity* Before Inoculation	Activity* 24-48 Hours After Inoculation	Activity* 2 Weeks After Inoculation	Mean Percent Change (\pm SE)	t Value for Difference Between Mean Percent Changes†; Degrees of Freedom; and Level of Significance
%						
Alkaline phosphatase‡	Ill	327 (234-588) (3)	155 (72-262) (4)	285 (188-449) (4)	-49.3 (\pm 7.2)	$t = 5.48$ 5 df; $P < 0.01$
	Not ill	356 (306-470) (3)	367 (276-475) (3)	327 (390-275) (2)	+1.4 (\pm 4.3)	
Sucrase§	Ill	49 (34-70) (2)	28 (13-43) (4)	56 (40-82) (4)	-42.1 (\pm 15.6)	$t = 1.80$ 5 df; $P = 0.13$
	Not ill	52 (39-79) (3)	53 (31-83) (3)	49 (36-66) (2)	-5.2 (\pm 10.5)	
Trehalase§	Ill	10.3 (8.1-13.0) (2)	4.5 (2.3-13.4) (4)	12.7 (7.8-19.3) (4)	-61.7 (\pm 10.5)	$t = 3.37$ 5 df; $P = 0.02$
	Not ill	24.7 (23.6-26.2) (3)	22.4 (15.1-28.1) (3)	27.2 (19.0-38.9) (2)	-16.2 (\pm 6.4)	

* Expressed as the geometric mean, with the range and number of volunteers studied in parentheses.

† Two-tailed t test.

‡ Expressed as micromols of p-nitrophenol per gram of protein per minute.

§ Expressed as micromols of glucose per gram of protein per minute.

microvillous height. These changes were associated with acute illness and were absent in both base-line and convalescence biopsies, as well as in volunteers who did not become ill.

Focal villous flattening and epithelial disarray are not specific intestinal lesions; they may be found in association with a variety of diseases and noxious stimuli, including gluten-sensitive enteropathy (22), irradiation (23), enterotoxins (24), pernicious anemia (25), and tropical sprue (26). Similarly, dilation of the endoplasmic reticulum, shortening of the microvilli, and widening of intercellular spaces may also be seen in several pathologic conditions of differing origins (27). However, in contrast to the intestinal lesions seen with invasive bacterial agents, such as *Shigella* (28) and enteropathogenic *Escherichia coli* (29), the mucosa remained intact in Norwalk-induced disease.

Rectal biopsies taken at the height of illness showed a normal histologic pattern. This evidence, along with the recent report of the absence of fecal leukocytes in Norwalk-induced disease (30), suggests that the colonic mucosa is relatively spared in this syndrome.

Jejunal brush-border enzyme activities (alkaline phosphatase, trehalase) were significantly decreased during the acute phase of illness but had returned to base-line levels by 2 weeks after illness. The decrease and time course of recovery are consistent with the transient histologic lesion of shortened and disrupted microvilli described above. Previously, transient malabsorption of D-xylose, fat, and lactose has been shown during acute illness induced by the Norwalk

agent; the malabsorption is no longer present at 9 to 11 days after acute illness (31). Therefore, definite but reversible pathophysiologic lesions are present in this viral-induced syndrome.

The intestinal lactase deficiency—a lactase level of less than 13 units per gram of protein and a sucrase to lactase ratio of greater than 4 (32)—was found in three of seven volunteers. Lactase deficiency in itself, however, did not appear to predispose to illness with the Norwalk agent, since one of three lactase-deficient volunteers did not become ill. In volunteers who became ill the base-line trehalase values were lower than those of a group of asymptomatic controls whose values ranged from 16 to 28 μ moles glucose/g protein-min. Volunteers who did not become ill, however, had levels that were within limits of the control values. Because such a small number of subjects was studied, the significance of this observation is unclear. However, the relative changes of enzyme levels between ill and well volunteers remain distinct.

Jejunal enzyme activity is decreased in a variety of conditions that affect the jejunal epithelium, including nontropical sprue (33), irradiation (23), pernicious anemia (25), infectious diarrhea in calves (34), feline infectious enteritis virus in cats (35), and staphylococcal enterotoxin gastroenteritis in rhesus monkeys (24). Improvement in brush-border enzyme activity parallels improvement in histology in pernicious anemia treated with vitamin B₁₂ (25), in nontropical sprue on a gluten-free diet (33), and in recovery from radiation (23) and enterotoxin (24). Investigations of the activity of other intestinal enzymes, such as adenyl cyclase and Na-K-dependent

ATPase, as well as the possible roles of enzyme alterations in the pathogenesis of acute infectious nonbacterial gastroenteritis, are currently under way.

The frequency of the disease, in four of seven volunteers, and the course of the illness produced in our experiments is consistent with that previously described with the Norwalk agent (10). All volunteers were clinically well by 48 hours after the onset of illness, without treatment or known sequelae.

Our study shows definite but reversible histologic and biochemical alterations in the human small intestine during acute infectious nonbacterial gastroenteritis. These pathophysiologic lesions are consistent with the clinical symptoms and time course of illness. Further studies with the Norwalk agent, as well as with more recently detected agents*, with both in-vivo and in-vitro experimental systems, should provide additional information about the pathogenesis of acute infectious nonbacterial gastroenteritis.

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