STUDIES ON THE PATHOGENESIS OF CHOLERA

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Recent interest in the pathogenesis of cholera has two origins. First, Asiatic cholera continues to ravage some of the world's most populous areas and with increasing use of air travel cholera is making its appearance in regions previously untouched, particularly, equatorial Africa. Since the simple expedient of separating sewage from drinking water that lead to the eradication of cholera from Europe and North America is unlikely, in the visible future, to become operative where cholera is endemic, other measures to effect its containment are being sought. The second origin of renewed interest in cholera is the development of animal models that faithfully reproduce the abnormalities associated with cholera in man.[1, 2] Not only has this advanced our knowledge of cholera but has been a useful prototype for the study of diarrheal diseases in general and has called attention to an all but forgotten aspect of intestinal physiology, intestinal secretion.

The volume of one patient's fecal fluid during an episode of cholera is shown in Figure 1. The patient, a porter, was working only a few hours before his admission to the hospital in shock and severe acidosis. His clinical state was attributable solely to the massive fluid and electrolyte loss caused by the acute voluminous diarrhea of cholera. During his five day illness, he lost 34 liters of fluid, an amount approaching the volume of his total body water.

What is the origin of this massive outpouring of fluid? It has been shown in man and experimental animals that it is produced by the small intestine with negligible contributions by stomach, liver, pancreas and colon. The fluid is isosmotic with plasma. Its electrolyte composition varies with the level in the intestine sampled and species studied, but, more important, it is similar to the normal intestinal fluid for that species at that level. The protein content of this fluid is very low, 85 mg./100 ml., well below the levels found in congestive failure and inflammatory bowel disease. The electrolyte composition of the "rice water" stools of cholera is similar to ileal fluid (Na^+139, $K24^+$, Cl^-106, HCO_3^-48 mEq/L). Although the absorptive func-

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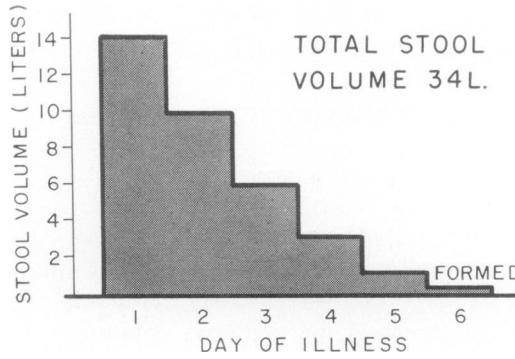

FIG. 1. Daily stool volumes from 38 year old man during the course of Asiatic cholera. After recovery his weight was 43 kg. (patient material through the courtesy of Dr. John G. Banwell).

tion of colon is apparently normal, it is limited and is unable to cope with the massive fluid and electrolyte load presented by the small intestine. Hence, there is relatively little loss in volume or change in electrolyte composition during transit through the colon.[3]

Over the years that cholera has been studied, four major suggestions have been proposed to account for the massive fluid and electrolyte losses: (1) Exudation of fluid secondary to denudation of the epithelium; (2) Increased filtration of fluid due to increased epithelial permeability; (3) Impaired intestinal absorption and (4) Increased secretion.

Exudation. In view of the impressive evidence to the contrary it is surprising that this view of Virchow and Koch on the pathogenesis of intestinal fluid loss in cholera persisted so long. In 1882, Cohnheim pointed out that the events in cholera could not be due to denudation of the intestinal epithelium because the choleraic stool has such a low protein content that it could not possibly arise from exudation.[4] Goodpasture, in 1923, added extensive histologic evidence that the intestinal epithelium was intact in cholera. Nevertheless, the notion that a morphologic alteration of the intestinal mucosa was a primary factor in the pathogenesis of cholera was not finally laid to rest until Gangarosa *et al.* found normal intestinal mucosa by peroral biopsies during cholera.[5] Animal models of cholera have made it possible to follow intestinal morphology from the first contact of the mucosa with V. cholerae or its exotoxin.[2, 6] These and other studies show that the cholera organism remains in the intestinal lumen and produces the pathophysiologic changes leading to cholera without invading or damaging the mucosa. In fact, the entire sequence of events can be produced by a cell-free filtrate of a V. cholerae culture containing only exotoxin. In spite of the transfer of large amounts of fluid from mucosal capillaries to intestinal

lumen, no morphologic changes in intestinal epithelium, vascular or lymphatic vessels, could be found. These studies, as well as evidence from the study of the disease in man, indicate that the intestinal fluid loss in cholera is the consequence of a functional rather than an anatomical alteration in the intestinal mucosa.

Increased Epithelial Permeability. It has been suggested that cholera exotoxin produces intestinal fluid loss by increasing the permeability of the intestinal epithelium, a suggestion compatible with the observed low protein content of intestinal fluid.[7] On the other hand, for increased epithelial permeability to lead to the movement of fluid into the intestinal lumen, a driving force must be applied across the epithelium, either an osmotic or hydrostatic gradient. In cholera in man, diarrhea continues even when the oncotic pressure of plasma has increased due to dehydration and the patient is in shock due to hypovolemia. In other words, intestinal fluid production continues in spite of striking decrease in oncotic and hydrostatic driving forces. In experimental cholera, mesenteric blood pressure was decreased to 30% of control values without altering the rate of intestinal fluid production.[8] In addition, increased permeability alone cannot account for the different anion concentrations found in jejunal and ileal fluid, i.e. in the jejunum, bicarbonate concentration is lower and chloride concentration is higher than plasma concentrations, whereas in the ileum, bicarbonate is high and chloride low.[9] Finally, when the ratios of isotopically-labelled uncharged solutes of differing molecular size such as, tritiated water, ^{14}C urea, ^{14}C creatinine and ^{14}C lactose, appearing in intestinal fluid after intravenous injection are measured, it was found that there is no difference between control and cholera animals although the direction of net fluid movement is opposite in the two groups.[10] Thus, neither direct nor indirect evidence supports the suggestion that an alteration in intestinal permeability is a factor in the pathogenesis of cholera.

Impaired Intestinal Absorption. Using Visscher's calculations for bidirectional rates of movement of sodium into and out of the intestinal lumen, Watten, et al. suggested that the diarrhea of cholera might be explained by an inhibition of sodium reabsorption in the face of continued normal transfer of sodium accompanied by water and electrolytes into the lumen.[11] Support for this notion was provided by the observation that crude cholera toxin inhibited active sodium transport by frog skin.[12] It has been estimated, however, that the small intestine normally absorbs 7–8 liters of fluid per day. Since in cholera there is no increased secretion of saliva, bile, pancreatic or gastric juice, complete inhibition of intestinal absorption would result in a 24 hour stool volume no greater than 7–8 liters. On the other hand, the average stool volume in the first day of cholera is 8.3 liters and volumes as high as 16 liters have been measured.[13]

Intestinal absorption of glucose is unimpaired in experimental cholera.[14] Since glucose and sodium absorption are coupled, it is unlikely that there is an inhibition of sodium absorption. In fact, measurement of unidirectional sodium fluxes in cholera in man and experimental animals has shown a normal lumen to mucosa movement of sodium while the flux into the lumen is greatly increased.[15, 16] The observation that glucose and associated sodium and fluid absorption are intact in cholera has been put to practical use by the oral feeding of glucose-electrolyte solutions, thus greatly decreasing the requirement for intravenous fluid and electrolyte replacement in cholera.[17, 18]

Increased Secretion. In recent years, there has been a tendency to view the intestinal mucosa as a homogeneous membrane much like frog skin or toad bladder. Some *in vitro* mucosal preparations indeed may behave in a similar fashion but it should be remembered that the intestinal mucosa is composed of several cell types arranged into crypts and villi each with different biochemical and physiological characteristics. Although present day physiologists largely ignore or doubt the existence of intestinal secretion, their predecessors did not. In their review of intestinal secretion in 1941, Florey et al. suggested, "It may be necessary for a constant secretion of fluid to take place from the crypts of Lieberkühn to keep food particles in suspension while they are attacked by pancreatic enzymes, and as the products of digestion are absorbed water and salts go with them. One may envisage a circulation of fluid during active digestion, the secretion passing out from the crypts of Lieberkühn into the lumen and back into the villi.[19]"

It is of interest that two famous students of cholera in the nineteenth century postulated that fluid production by the intestine in cholera originated from increased intestinal secretion. Dr. John Snow in 1855 wrote, "It would seem that the cholera poison, when produced in sufficient quantity, acts as an irritant on the surface of the stomach and intestine, or, what is still more probable, it withdraws fluid from the blood circulating in the capillaries, by a power analogous to that by which epithelial cells of the vaious organs abstract the different secretions in the healthy body.[20]" After presenting extensive clinical, pathological, and experimental data, Cohnheim presented a similar view, "... the process of cholera may be interpreted by supposing that first, under the influence of the virus, which has probably entered the intestine from without, there takes place, an extraordinary profuse secretion from the glands of the small intestine.[4]"

Since glucose absorption, a function of the villi, is intact in cholera and studies of bidirectional fluxes of sodium have shown that movement from lumen to blood is normal while movement into the lumen is increased, it seems important to reexamine Cohnheim's hypothesis. If "absorption" and "secretion" are anatomically separated, cholera exotoxin might stimulate the "secretory area" and leave the "absorptive area" unaffected. Thus, if

cholera exotoxin stimulates secretion by the crypts of Lieberkühn, agents which damage the crypts preferentially would be expected to modify the secretory response to cholera toxin. Observations with cycloheximide, an inhibitor of protein synthesis supported this suggestion. On exposure to cycloheximide the epithelial cells of the crypts of Lieberkühn, having a higher protein synthetic rate, are affected earlier and at a lower dose than the absorptive columnar cells of the villi. The first detectable morphologic evidence of this reversible inhibition of protein synthesis is the disappearance of mitotic figures from the crypts. With increasing doses of cycloheximide, the crypt epithelium shows irreversible damage, and at still higher does, the epithelium of the villi show morphological and functional damage. At a level causing inhibition of mitosis, cycloheximide pretreatment inhibits the outpouring of fluid and electrolytes that normally follows exposure of the intestinal mucosa to cholera toxin.[21] Calculations of bidirectional fluxes indicate that the cycloheximide effect is due entirely to inhibition of the cholera toxin-induced increase in blood to lumen flux.[22]

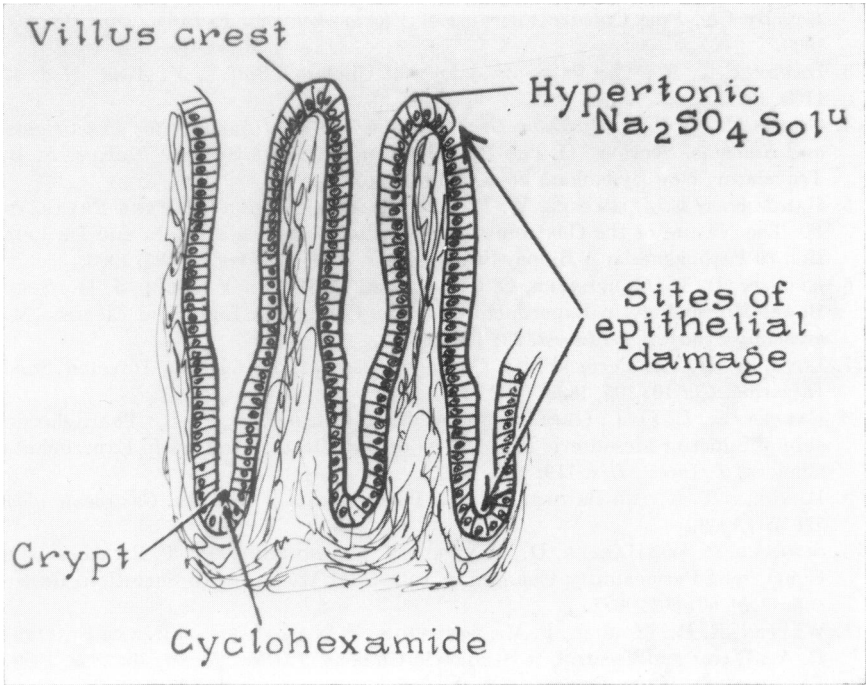

FIG. 2. Cycloheximide, in a dose that damages the crypts but does not alter glucose absorption, prevents the intestinal secretion induced by cholera exotoxin. Damage to the villus crests by exposure to hypertonic sodium sulfate decreases glucose absorption without altering secretory response to cholera exotoxin.

The corollary of the hypothesis that cholera fluid originates from the crypts would be to show that damage to the absorbing epithelium did not interfere with the intestine's response to cholera toxin. It was found that exposure of the intestine to hypertonic sodium sulfate (2100 mOsm for 30 min.), while producing damage to the villous epithelium and decreasing glucose absorption did not alter the secretory response to cholera toxin (Fig. 2).[23]

The data presented suggest that the intestinal epithelial cells responsible for absorption are different than those involved in secretion. Cholera and many other diarrheal diseases may be viewed as hypersecretion of the crypts of Lieberkühn. The characterization of this secretory mechanism and its stimulation and control currently are the subjects of exciting research which it is hoped will lead to methods of limiting the devastating impact of cholera.

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DISCUSSION

DR. JAMES A. CLIFTON (Iowa City): Was the dose of cycloheximide that you used to effect the secretion the same as was shown on the photomicrograph? Secondly, in cholera, is there any change in the glycocalax or any change in disaccharidase activity?

DR. HENDRIX: The dose that was shown in the photomicrograph was 40 mg/kg and we used half that amount. I did not show the photomicrographs with 20 mg/kg because all that is seen is a decrease in mitotic figures. Studies about the glycocalax have not really been done. On the other hand, one of the ideas of what immunity to cholera is about is that the organisms do not stick or adsorb on to the epithelium in the immune animal where they do in the susceptible one. Exactly what that means as far as mechanism is concerned is not clear.

DR. JAMES W. HAVILAND (Seattle): Did the cycloheximide managed animals, I guess one could call them that, show any other effects from the cholera bacilli or from the toxin?

DR. HENDRIX: No, there were no other abnormalities seen with this dose and since the manifestations of cholera as seen in the experimental animal or man are the consequence of the loss of fluid and electrolytes, blocking that response blocks the clinical or experimental manifestations.

DR. GEORGE SCHREINER (Washington): A beautifully organized study, Tom.

I'm curious about the lesion which you stated was irreversible with the cycloheximide 40 mg dose. What happens in the evolution of this lesion? And what happens if you challenge these animals later on? Do they retain a permanent immunity aganst the secretory aspects of cholera toxin?

DR. HENDRIX: With a dose as large as that they don't live long. If you damage the crypts irreversibly, the source of the new cells is destroyed and soon the epithelium is completely denuded and the animal succumbs from infection. On the other hand, if one gives a smaller dose of cycloheximide such as I used, and then repeatedly challenges the animal with cholera toxin, he is not responsive to toxin for 12 to 14 hours after giving cycloheximide. Then as a shower of mitotic figures appear, the mucosa again becomes responsive to cholera toxin with outpouring of fluid.

DR. DANIEL S. ELLIS (Boston): Do you have any idea whether the effect of cycloheximide is something that is specific in cholera or whether it also might be effective in other diseases in which there is an abnormality of secretion?

DR. HENDRIX: It is not limited. I think this is a non-specific function. The intestine probably responds with secretion in response to a lot of stimuli. For example, the response to hypertonic osmotic gradiant across the mucosa, though not prevented by cycloheximide, can be cut in half. The response to E-coli toxin, which is similar to cholera toxin, is also blocked.

DR. FRANK P. BROOKS (Philadelphia): Tom, do you envision these cells as secreting exclusively water and electrolytes? Is there any evidence that any larger molecules might be contributed?

DR. HENDRIX: The crypts themselves have a very heterogenous population of cells and some of them clearly look to be secretory. So I think that there are many things that come out of the crypts. The response to cholera toxin is primarily water and electrolytes, but as you saw, the goblet cells also are discharged, so goodness knows what else may be coming out in addition to the mucus.

DR. FRANCIS C. WOOD (Philadelphia): Tom, you didn't answer the question I thought you were going to. Have you treated any patients with cholera with cycloheximide in the proper dose?

DR. HENDRIX: No, in fact I am not sure that I would want to recommend that. I think it gives a model for agents that do interfere with this secretory process that might be used to cut down acutely on secretory response and thereby limit the requirements for intravenous fluids. This is really the thing that is of practical importance in the areas where cholera is endemic.

DR. BELTON A. BURROWS (Boston): This is not intended as a facetious question. In this day and age of recycling everything from tin cans to cellophane baggies, etc., have animals ever been maintained by recycling their secretions?

DR. HENDRIX: Not that I know of. On the other hand, the fluid that one infuses is designed to mimic what they actually lose, and what they lose is an electrolyte solution which is isotonic with plasma, with similar potassium and sodium concentrations, high bicarbonate and low chloride. But to strain out all the other things that might be in the intestinal contents and put that back in intravenously, I think that would be another ball game.

DR. HARVEY: Of course, other advantages of nature are available. The juice of of green coconuts, known as *dab* has the same electrolyte content as human blood including adequate amounts of potassium. Furthermore it's sterile—and sterile intravenous fluids are hard to get in that part of the world. Coconut juice has been used as a substitute for the types of electrolyte and water solutions that we use here.