The Production of Intestinal Fluid by Cholera Toxin in the Rat[1] (36444)

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(Introduced by S. F. Marotta)
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The diarrheagenic toxin produced by *Vibrio cholera* causes an enormous loss of fluid through a histologically normal intestinal epithelium. This has been hypothesized to be a result of: (A) an absorption failure (1), (B) an increase in intestinal permeability (2) or an increase in filtration of capillary plasma (3), or (C) an increase in secretion by epithelial cells (4, 5). The first hypothesis seems the least acceptable since the flux of sodium (Na) from lumen to blood is unaffected by cholera toxin (6, 7). The second hypothesis is proposed despite the presence of normal intestinal morphology following cholera toxin exposure (4, 8–11). The third hypothesis is based upon the assumption that the toxin may stimulate the production of a hormone which activates secretory mechanisms. The ability of cycloheximide, a protein synthesis inhibitor, to depress the production of fluid caused by cholera toxin supports this concept (6). Inhibition of carbonic anhydrase activity has also been reported to depress the amount of fluid produced by this toxin (12).

The present investigation was designed to determine: (A) if the rat could serve as a suitable model to study the pathogenesis of cholera, and (B) if the secretion induced by cholera toxin *in vivo* could be demonstrated in intestinal segments *in vitro* taken from animals showing the pathological effects of the toxin. An earlier study reported no significant differences between control and experimental groups when cholera toxin was added directly to an *in vitro* preparation (13).

Methods. Male Holtzman rats, weighing 280 to 300 g, were used. Food was withheld and they were given 5% glucose in water to drink *ad libitum* for 24 hr before use. While under ether anesthesia a segment of small intestine 40 cm in length was measured beginning at the distal end of the ileum. A ligature was placed around the proximal end of the segment at the nearest midpoint between two branches of the mesenteric artery. With the proximal end closed the luminal contents were flushed into the caecum with 10 ml of 0.9% saline which was introduced with a syringe and 27 gauge needle. The segment was emptied by gentle stripping and then the distal end was ligated. In some studies a 40 cm segment of intestine was measured proximal to the initial distal 40 cm segment and loops were prepared in the same manner. Loops of the duodenojejunal portion of the small intestine and loops of the jejuno-ileal portion of the small intestine are referred to hereafter as proximal and distal loops, respectively. The empty loops were filled with 3 ml of a solution (pH 7.55) containing 10, 20 or 30 mg/ml Wyeth cholera toxin (NIH Lot 001) supplied by the NIH Cholera Advisory Committee. In control loops the toxin was replaced by an equal volume of a 10, 20 or 30 mg/ml Bacto-Peptone broth (Difco) solution and the pH was adjusted to 7.55. To these aqueous solutions, sodium chloride was added (if necessary) to attain an osmolality of 300 mOsm. Five hours later, under ether anesthesia, the loops were removed and weighed with and without their contents. Cycloheximide (20 mg/kg) and acetazolamide (Diamox-parenteral, 50 mg/kg) when used were given intravenously 20 min before the preparation of the loop and an equal amount of the same drug was placed in the loop. One group of

[1] Supported in part by PHS GRSG No. 370.

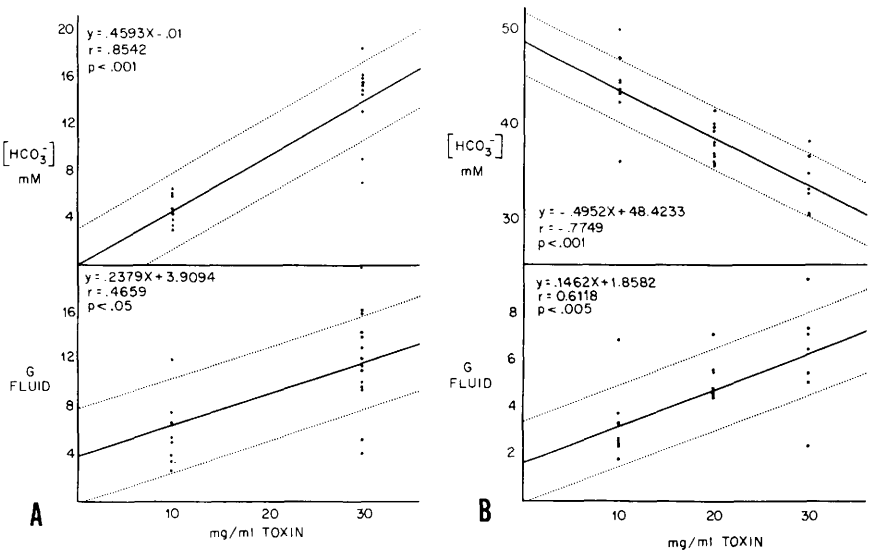

FIG. 1. Fluid production (g) and bicarbonate concentration (m*M*) in the proximal (A) and distal (B) loops of the rat small intestine following the *in vivo* administration of cholera toxin (mg/ml). Each point is an observed value and the line was calculated by the least square method.

rats was given 20 mg/kg cycloheximide iv without subsequent preparation of loops and the gastrointestinal tract was examined 90 min later to determine the ability of this drug to cause fluid accumulation in the intestinal tract.

The *in vitro* studies consisted of preparing distal loops with toxin *in vivo* and then removing them 4 hr later. They were everted and cannulated according to a method described previously (14, 15) and incubated at 37° in a tube containing 50 ml Krebs–Ringer bicarbonate solution, made up according to Umbreit, Burris, and Stauffer (16). All solutions were aerated with 95% O_2 and 5% CO_2. This procedure permitted the use of the same segment for investigation over several sequential periods of 1 hr. Two sacs were prepared from each animal. One milliliter of the Krebs solution containing 175 mg inulin/100 ml as a water marker, was placed in the everted sac (serosal solution). Both mucosal and serosal solutions were changed at the end of each period. A second *in vitro* method employing closed everted sacs prepared from *in vivo* loops 4 hr after the administration of cholera toxin was also used to study fluid transport. They were incubated as the others in Krebs–Ringer with 0.5 ml of the same solution placed inside the sac. After a preincubation of 30 min they were weighed and then reweighed with and without their contents after incubating for an additional hour.

Na and potassium (K) were determined by flame photometry (IL Model 143) and inulin was measured colorimetrically (17). Chloride (Cl) was determined with a chloridometer (Buchler-Cotlove), and bicarbonate (HCO_3) by an electrotitrimetric method employing an IL Model 245 pH meter (18). Osmolality was estimated by freezing point depression with a Precision Instrument Osmette. The use of inulin as a water marker in one of the *in vitro* methods has been previously reported (14, 15) and the calculations are as follows:

$$\text{Fluid movement: } Q_{H_2O} = \frac{\text{initial inulin conc}}{\text{final inulin conc}} - 1 = \text{ml fluid transported/hr/sac};$$

$$\text{Net Na transport: } Q_{Na} = (Q_{H_2O} + 1)\ (\text{final Na conc}) - (\text{initial Na conc}) = \mu\text{Eq Na transported/hr/sac.}$$

All values are expressed as means ± standard error and statistical significance was computed according to the Student's *t* test.

Results. Five hours after the intestinal

TABLE I. Ion Concentrations in Intestinal Fluid and Plasma.

Group (toxin conc)	Source	mEq/liter Na	K	Cl	HCO_3	Osmolality (mOsm)
Proximal	Fluid	144.6[a]	5.5	133	4.9	289.7
10 mg/ml		(1.2)	(0.1)	(1.7)	(0.4)	(4.0)
	Plasma	146.0	4.3	101	21.7	311.1
		(0.5)	(0.2)	(0.3)	(0.1)	(4.5)
30 mg/ml	Fluid	150.5	5.5	126	15.0	299.8
		(1.2)	(0.1)	(2.0)	(0.8)	(5.2)
	Plasma	146.6	5.0	100	21.0	311.1
		(0.8)	(0.1)	(0.3)	(0.1)	(4.6)
Distal	Fluid	144.2	5.9	89	34.1	288.7
30 mg/ml		(1.0)	(0.1)	(2.8)	(1.3)	(3.7)
	Plasma	142.3	4.4	102	20.1	304.1
		(0.6)	(0.2)	(0.3)	(0.1)	(5.0)

[a] Mean ± SE in parentheses.

loops were prepared, there was no fluid in the control loops but a substantial amount accumulated in the toxin treated loops (Fig. 1). The production of fluid in distal loops was dose-dependent over the range of 10 to 30 mg cholera toxin/ml (Fig. 1B), and the distension of the loops with fluid following the lowest toxin concentration was apparent upon opening the abdomen. Although only 2 concentrations of toxin were used in proximal loops, it appears that fluid production here may also be dose-dependent (Fig. 1A). Fluid production in the proximal loops was approximately twice the amount observed in distal loops. Wet tissue weight, following toxin, of empty distal and proximal loops were 3.63 ± 0.17 and 4.57 ± 0.21 g, respectively, and there were no significant differences in the tissue weight among the toxin doses. Wet tissue weight of control loops were 3.55 ± 0.11 and 3.65 ± 0.12 g for the distal and proximal loops, respectively. The compositions of intestinal fluids produced by cholera toxin and of plasma from experimental animals are summarized in Table I. The concentrations of HCO_3 in loop fluid was inversely proportional to the concentration of toxin used and to the amount of fluid produced in distal loops, while it was directly proportional to these 2 parameters in the proximal loops (Fig. 1).

Acetazolamide did not significantly influence the accumulation of fluid produced by toxin (Table II). In control animals treated with this drug, no fluid was present in the loops after 5 hr. Wet tissue weight of both control ($p < .001$) and toxin ($p < .02$) loops following the injection of acetazolamide were significantly less than loops from similarly treated animals not given acetazolamide. Cycloheximide also did not reduce fluid production in toxin-treated loops, while in the control animals it resulted in a greater production of fluid ($p > .10$). In these 2 groups there was always a marked distension of the entire bowel with fluid, while in all other groups fluid accumulation occurred only in the loops. Five rats, given cycloheximide (20 mg/kg, iv) and not subjected to toxin or loop preparation, showed marked fluid accumulation throughout the entire gastrointestinal tract within 90 min. The ionic composition of intestinal fluid following this drug was not significantly different from plasma, in all 3 groups.

In the first *in vitro* experiment, which studied the net transport of Na and fluid in cannulated everted sacs, there was no significant difference between the amounts transported in sacs prepared from experimental (treated with toxin *in vivo*) and control loops, except during the second hour (Fig. 2). The amounts of Na and fluid transported in the first hour were less than in the subse-

TABLE II. The Effects of Acetazolamide and Cycloheximide on the *in Vivo* Production of Fluid in Distal Intestinal Loops 5 hr After Cholera Toxin (10 mg/ml).

Drug	Group	No. of rats	Wt (g)		Fluid transported (g/g tissue)
			Tissue	Fluid	
None	CT[a]	20	3.35 ± 0.15[b]	3.31 ± 0.40[c]	0.99 ± 0.04
	C	20	3.02 ± 0.10	0	0
Acetazolamide	CT	8	2.83 ± 0.11[d]	3.86 ± 0.39[c]	1.36 ± 0.14
	C	8	2.56 ± 0.06[d]	0	0
Cycloheximide	CT	9	3.66 ± 0.09	3.43 ± 0.34	0.94 ± 0.09
	C	8	3.64 ± 0.08	4.36 ± 0.43	1.19 ± 0.12

[a] CT = cholera toxin; C = control.
[b] Mean ± SE.
[c] $p < .001$ (CT vs C).
[d] $p < .05$ (CT vs CT and C vs C in none and acetazolamide groups).

quent 2 hr, but this was significant ($p < .05$) in the control group only.

In the second *in vitro* experiment, where determination of fluid transport in closed everted sacs was made by weight measurements, there was no significant difference in wet tissue weight between 20 control and 20 experimental (treated with toxin *in vivo*) sacs (1.80 ± 0.06 vs 1.99 ± 0.08 g) nor was there any difference in the amount of fluid accumulated in these sacs (0.18 ± 0.02 vs 0.16 ± 0.02 g fluid transported/g tissue/hr). In both groups this accumulation of fluid was different from zero ($p < .001$).

Discussion. Cholera toxin readily stimulated the production of intestinal fluid in both the proximal and distal half of the small intestine; however, the rate of fluid production was twice as great in the former as in the latter. This indicated that the upper small intestine was more sensitive to the action of cholera toxin and may be a better region for studying the pathogenesis of this disease. It is suggested that this difference is due to the greater permeability of the mucosal membranes, as expressed with a greater estimated membrane-pore radius, in the proximal portion (19). The ionic concentrations of both proximal and distal loop fluid were similar to normal secretions in each region. In both loops as fluid production increased, the concentration of HCO_3 and Cl in intestinal fluid changed and approached those in plasma while the Na and K levels remained unchanged. This would not occur if either a mechanism for active secretion of Cl or HCO_3 were the major means of fluid production in cholera, but the opposite direction of Cl and HCO_3 changes would occur with increased fluid production in each region. This suggests that the fluid produced in the intestine following cholera toxin is a filtrate of plasma.

It has been reported that both acetazolamide (12) and cycloheximide (6) minimized the production of fluid induced by cholera toxin in the rabbit. However, carbonic anhydrase inhibitors which are known to depress fluid and electrolyte absorption in the rat jejunum (20), should enhance the accumulation of fluid in the bowel. Acetazolamide did not inhibit the production of fluid in loops of rat intestine treated with cholera toxin, and although this drug inhibits fluid and electrolyte absorption in the intestine it had no effect on the complete absorption of fluid from loops without toxin over a 5 hr period. Since this carbonic anhydrase inhibitor did not minimize toxin-induced fluid accumulation in the rat it is unlikely that carbonic anhydrase activity is involved in a hypersecretion of HCO_3 which in turn has been suggested as one of the means of secretion to produce the intestinal fluid induced by cholera toxin in the rabbit (12). The presence or absence of carbonic anhydrase activity would not affect the cholera toxin-induced production of intestinal fluid if the

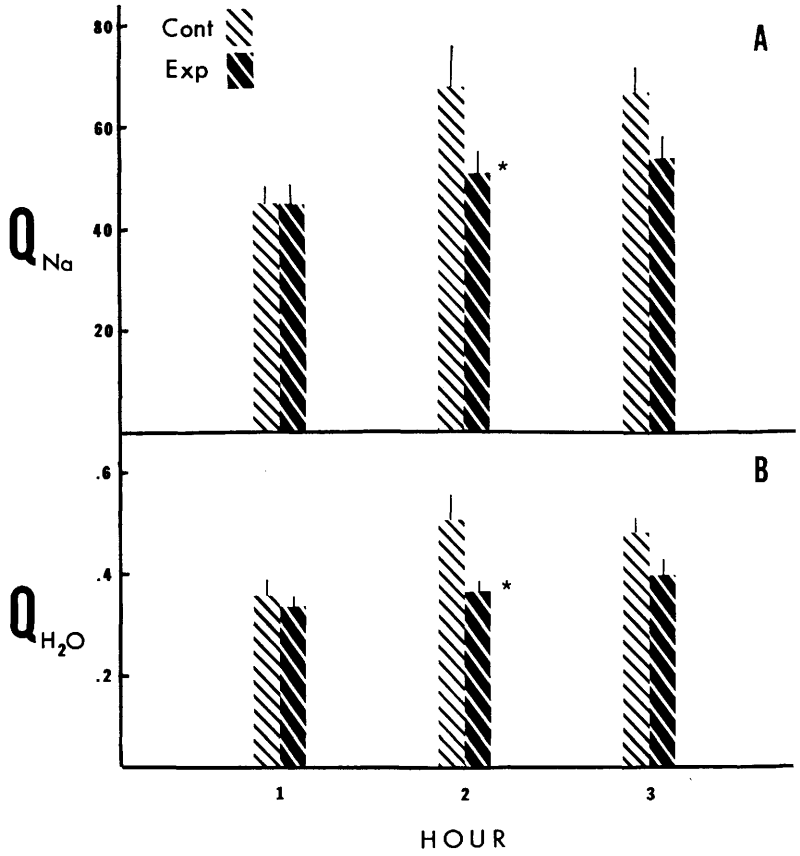

FIG. 2. Net transport of Na (A) and fluid (B) into cannulated everted sacs (20 control and 20 experimental) prepared from 10 control (Cont) and 10 cholera toxin (Exp) loops of distal intestine. Q_{Na} = μEq Na/hr/sac, Q_{H_2O} = ml fluid/hr/sac (both mean $\pm$ SE) * difference from control is significant $p < .05$.

process were one of filtration of capillary plasma and not ion secretion by the mucosa. Wet tissue weight following acetazolamide administration was significantly reduced. This finding agrees with another report which shows that the decrease represents a difference in water content (20).

The attenuating effect of a protein synthesis inhibitor, cycloheximide, on intestinal fluid production during cholera toxin administration in the rabbit has led some to suggest that the synthesis of a hormonal secretagogue is involved in the pathogenesis of cholera (5). This drug produced a greater accumulation of fluid than the cholera toxin in *in vivo* loops, which is different from its action on cholera loops of intestine in the rabbit. This antibiotic may depress Na and fluid transport from the lumen of the intestine via its inhibitory action on protein synthesis. If this occurs, the rapidity of its onset of action (marked fluid accumulation in 90 min) indicates that it must inhibit synthesis of protein structures that have high turnover rates. It is possible that this drug decreased the net transport of fluid out of the intestine by increasing the permeability of epithelial cells, by an effect unrelated to its action on protein synthesis. The results of the cycloheximide study are in accord with the theory of active hypersecretion since the accumulation of fluid produced by this drug masks any effect it may have when the toxin is added. Presently it is not possible to determine whether the effects of cholera do or do not contribute to fluid accumulation induced by

cycloheximide.

It has been suggested that increased intestinal permeability may occur in the pathogenesis of cholera (2). The concept that permeability may be affected is consistent with the findings that a lipase stimulating factor is present in cholera toxin (21). This prompted the use of two different *in vitro* methods in this study. Inulin is used as a water marker in one method and if intestinal permeability is increased by cholera toxin, this could result in the loss of inulin from the everted sac which would give erroneous results if its loss was disproportionate to the loss of fluid. Problems in using this marker can be obviated by weighing everted sacs to measure the accumulation of any fluid, and this was done with the second *in vitro* method in this study. The latter method (employing closed sacs), confirming results of the first experiment (employing cannulated sacs), indicated that permeability was not affected since fluid and electrolytes accumulated in the closed everted sacs. It was reported earlier that this cannulated sac preparation maintains its function to transport fluid and electrolytes to the serosal side for more than 3 hr and net fluid and sodium transport are in a steady state in the second and third hours (15). This is confirmed by the control sacs in this study.

With less elaborate preparation than reported by others (22, 23), the rat can be used to study the pathogenesis of cholera. The intestinal loops can be prepared rapidly and with relative ease, which coupled with availability and relatively low cost of rats make the rat a cholera model which could be used in future investigations. It is comparable to rabbit ileal loops in its sensitivity to toxin, however, assay methods based on lipolysis in fat cells and on skin permeability are more sensitive to cholera toxin (21). It is considered that the results of this study support the hypothesis that an increased filtration from the capillary plasma is the most important means of intestinal fluid production in the pathogenesis of cholera. No evidence was found for an active secretory process in epithelial cells to produce intestinal fluid in cholera. The rat cholera model presented here is quite different from that of the rabbit. It is important to determine which one is more like the pathogenic process in the human.

Summary. The intestine of the rat can be used as a cholera model. The amount of fluid production in *in vivo* intestinal loops was dependent on the amount of toxin present. With increased fluid production HCO_3 and Cl concentrations approached those in plasma. Acetazolamide did not minimize fluid production while it was uncertain whether cycloheximide had any effect. In *in vitro* everted sacs, prepared from intestines which were secreting fluid in response to cholera toxin *in vivo*, there was a net transport of fluid from the mucosal to serosal side.

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Received July 26, 1971. P.S.E.B.M., 1972, Vol. 140.