Effect of *Vibrio cholera* Toxin on Vascular and Extravascular Spaces of Rat Intestine (36718)

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The toxin produced by *Vibrio cholera* causes the secretion of massive amounts of fluid in the small intestine. The precise mechanism of action of this toxin is unknown; however, many investigations have shown that it produces no inflammatory changes and only minor morphological changes in the intestinal mucosa (1-5). For this reason, the loss of body fluids during cholera is considered to be mediated initially by functional alterations of the intestinal mucosa. An early event in the pathogenesis of cholera is the activation of adenyl cyclase to increase cyclic adenosine monophosphate (cAMP) levels (6, 7). Increasing cAMP levels by either cholera toxin or drugs (*e.g.*, theophylline) causes the electrogenic secretion of chloride and inhibition of the active absorption of sodium in *in vitro* segments of ileal mucosa (8). On the other hand, others have reported that cholera toxin *in vivo* increases the plasma to lumen flux without affecting the lumen to plasma flux of sodium (9). Previous studies from this laboratory have suggested that cholera toxin causes a hypersecretion of intestinal fluid by increasing the filtration of fluid from the capillary plasma into the lumen of the intestine (10, 11). This is in contrast to a theory that the fluid is produced by activation or inhibition of mucosal cell secretory and absorptive processes (12).

Capillary dilation (4, 5), edema of the lamina propria (4, 5), and rarefaction of the capillary endothelium (5) have been reported to occur in the mucosal layer of the intestine following exposure to cholera toxin. In addition, increased capillary permeability has been suggested as the cause of the marked edema that follows intradermal or intramuscular injection of the toxin (13, 14). The purpose of this study was to determine if the application of cholera toxin to the luminal surface of the intestinal muscosa changes capillary permeability, since mucosal capillaries are normally very fenestrated (15) and thus poorly restrict the movement of serum albumins into the extravascular space (16). This study reports the effect of cholera toxin on the volumes of distribution of red blood cells, serum albumin, dextran, inulin and urea in intestinal tissue.

Methods. Male Holtzman rats (300 g) were used and restricted to a diet of 5% glucose solution for 24 hr before the experiments. Sodium iodide 0.01% was added to the drinking solution for 36 hr prior to the experiments in which radioiodinated (^{125}I) serum albumin (RISA-125) was injected. This was done to minimize retention of any ^{125}I freed from metabolized serum albumin (17). The rats were anesthetized with ether and a 40 cm segment of upper small intestine was measured off beginning 15 cm from the pylorus. The proximal end of the segment was ligated at the midpoint of two branches of the mesenteric artery. The lumen of the segment was then flushed with saline by injecting 10 ml through a 27 gauge needle inserted through the intestinal wall. After emptying the loop by gentle manual expression, the distal end was ligated forming a closed loop which is referred to hereafter as a proximal loop. The saline flushed into the lower small intestine was emptied into the cecum and another 40 cm loop was prepared from this lower segment in the same manner. It is referred to hereafter as a distal loop. To prepare an experimental loop a proximal loop was injected with 3 ml cholera toxin solution

TABLE I. Serum Albumin-^{125}I and RBC-^{51}Cr Spaces (5 hr).

Loop section[a]	Expt.	Albumin in space (μl/g)	Albumin space (μl/g)	HCT (%)	Tissue H_2O (%)
Proximal	Control[b]	133 ± 6[c]	8.5 ± 1.4[c]	49 ± 0.6	77.1 ± 0.5
	Toxin[d]	186 ± 9[e]	11.0 ± 0.8	53 ± 0.8[e]	79.4 ± 0.5[e]
Distal	Control[b]	112 ± 6[f]	9.4 ± 0.8	49 ± 0.6	76.0 ± 0.4
	Control[d]	105 ± 7[f]	8.4 ± 0.6[g]	53 ± 0.8[e]	75.3 ± 0.4

[a] $N = 14$ for each group. Loop ^{125}I concn/plasma ^{125}I concn × 100 = 1.96 ± 0.3%.
[b] One proximal and one distal loop from same animal.
[c] Mean ± SEM; μl/g wet tissue wt.
[d] One proximal and one distal loop from same animal.
[e] Significantly different from controls $p < .05$.
[f] Significantly different from proximal controls $p < .05$.
[g] Significantly different from proximal toxin $p < .05$.

(30 mg/ml, Wyeth cholera toxin, NIH Lot 001). To prepare a control loop a proximal or distal loop was injected with 3 ml of Bacto-peptone solution (30 mg/ml, Difco) which is the broth used to prepare the lypholized crude filtrate containing cholera toxin. The pH and osmolality of the control solution were adjusted to that of the toxin solution. Anesthesia, surgery and loop preparation were not done in one group given RISA-125 to determine the effect of these procedures on the albumin space.

Radioactively labeled compounds were used to determine their volumes of distribution in intestinal tissue. They were given intravenously (iv) at the time of loop preparation except one group was given RISA-125 67 hr before to determine if its 5 hr space changes with time. Chromium-51 (^{51}Cr) was used to label rat red blood cells (RBC-^{51}Cr) following the manufacturer's (Abbott) directions. Inefficient labeling occurred and was corrected by removing the plasma and washing the RBC before labeling. RISA-125 (Abbott) was added to the RBC-^{51}Cr to give a hematocrit (HCT) of 35%. Aliquots (mean 875 mg, containing approx 3 μCi of each isotope) of this doubly labeled whole blood were injected iv and the volumes of distribution of RBC and serum albumin were measured. Five μCi of dextran-^{14}C (mol wt 60,000–90,000), inulin-^{14}C (mol wt 5000) and urea-^{14}C obtained from New England Nuclear were given separately iv to individual rats and the respective spaces were measured.

Blood and tissues from animals injected with ^{51}Cr and ^{125}I were counted in an autogamma scintillation counter (Nuclear-Chicago) and separation of ^{51}Cr and ^{125}I counts, when these isotopes were given together, was accomplished by pulse height analysis and standard isotope crossover correction techniques. Tissues with ^{14}C labels were homogenized and deproteinated with trichloroacetic acid. Plasma samples with ^{14}C labels were similarly deproteinated. Aliquots of these protein-free extracts were added to Instagel (Packard) and counted in a Beckman LS 100 liquid scintillation counter to an error of 1%. Corrections for quenching were made with the external standard ratio. Calculations of the sizes of the spaces were determined as follows:

$$\text{Red cell space } (\mu\text{l/g tissue}) = \frac{\text{mg blood/g tissue}^1}{1.056^2} \times \text{HCT},$$

$$\text{albumin space } (\mu\text{l/g tissue}) = \frac{\text{mg blood/g tissue}^1}{1.056^2} \times (1 - \text{HCT}),$$

$$\text{dextran, inulin and urea spaces } (\mu\text{l/g tissue}) = \frac{\text{epm/g tissue}}{\text{epm } \mu\text{l plasma}}.$$

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

[1] $\frac{\text{cpm/g tissue}}{\text{cpm/mg heart blood}}$.

[2] Sp gr of rat blood.

TABLE II. Serum Albumin-^{125}I Space (72 hr).

Loop section[a]	Expt.	Albumin space (μl/g)	HCT (%)
Proximal	Control[b]	137 ± 8[c]	48.2 ± 0.8
	Toxin[d]	186 ± 8[e]	53.7 ± 1.0[e]
Distal	Control[b]	122 ± 9	48.2 ± 0.8
	Control[d]	112 ± 14	53.7 ± 1.0
Proximal	No loop[f]	144 ± 12	49.8 ± 1.4
Distal	No loop[f]	136 ± 12	49.8 ± 1.4

[a] $N = 8$ for each group with loops, $N = 6$ for no loop group.

[b] One proximal and one distal loop from same animal.

[c] Mean ± SEM; μl/g wet tissue wt.

[d] One proximal and one distal loop from same animal.

[e] Significantly different from all other groups $p < .05$.

[f] Proximal and distal intestine in rats with no loops.

Results. The 5 hr albumin and RBC spaces are summarized in Table I. The albumin space in proximal loops treated with toxin was significantly greater than both proximal and distal control loops. The toxin also caused the per cent of water in these tissues to increase significantly. In this and all subsequent groups, the hematocrit of heart blood was significantly greater in the toxin treated rats than the controls. The RBC space increased in toxin treated proximal loops but not significantly. The concentration of ^{125}I in the loop fluid was only 1.93% of that in plasma. It was all precipitable with trichloroacetic acid. The albumin space at 72 hr was significantly increased by cholera toxin as was the hematocrit of the animals in this group (Table II). The albumin space at 72 hr was the same as at 5 hr in both control and experimental loops.

Although the dextran space (Table III) in toxin treated loops was not significantly different from controls, the percentage tissue water was significantly increased. The amount of dextran in the loop fluid was very small compared to plasma levels (2% of plasma). The inulin space, which ranged from 58 to 74% of the total water space was decreased in toxin treated loops (Table IV). The concentration of inulin in loop fluid was small compared to plasma levels. The urea space (Table V) in toxin treated loops was not significantly different from controls and was not significantly different from tissue water as determined by weighing wet and dried tissues.

In the toxin treated loops of all the groups a mean of 13.2 ± 0.30 g of fluid was produced in 5 hr while the control loops were always empty.

Discussion. The percentage water in intestinal tissues treated with cholera toxin increased significantly in all except the group given inulin as a tracer. Morphological studies showing edema of the lamina propria (4, 5) and epithelial intracellular vacuolization with hypertrophy of the smooth endoplasmic reticulum (5) suggest that this increase is confined to changes in the water content of the epithelial cell and the mucosal extracellular space. The dextran space which is assumed here to be entirely an extracellular space did not significantly increase following cholera toxin. This suggests that the most important part of the increase in tissue

TABLE III. Dextran-^{14}C Space (5 hr).

Loop section[a]	Expt.	Dextran space (μl/g)	HCT (%)	Tissue H_2O (%)
Proximal	Control[b]	209 ± 7[c]	49 ± 1.0	77.3 ± 0.4
	Toxin[d]	213 ± 14	57 ± 2.0[e]	79.0 ± 0.4[e]
Distal	Control[b]	214 ± 13	49 ± 1.0	76.1 ± 0.3
	Control[d]	226 ± 28	57 ± 2.0	75.5 ± 0.5

[a] $N = 6$ for each group. Loop dextran conen/plasma dextran conen × 100 = 2.01 ± 1%.

[b] One proximal and one distal loop from same animal.

[c] Mean ± SEM; μl/g wet tissue wt.

[d] One proximal and one distal loop from same animal.

[e] Significantly different from controls $p < .05$.

TABLE IV. Inulin-^{14}C Space (5 hr).

Loop section[a]	Expt.	Inulin space (μl/g)	HCT (%)	Tissue H_2O (%)
Proximal	Control[b]	586 ± 8[c]	46.2 ± 0.7	78.0 ± 0.3
	Toxin[d]	452 ± 19[e]	51.3 ± 1.4[e]	78.8 ± 0.3
Distal	Control[b]	538 ± 35	46.2 ± 0.7	75.6 ± 0.6
	Control[d]	551 ± 25	51.3 ± 1.4	75.0 ± 0.5

[a] $N = 6$ for each group. Loop inulin conen/plasma inulin conen × 100 = 5.0 ± 0.6%.
[b] One proximal and one distal loop from same animal.
[c] Mean ± SEM; μl/g wet tissue wt.
[d] One proximal and one distal loop from same animal.
[e] Significantly different from controls $p < .05$.

water content is due to an increase in the intracellular water content. The inulin space in toxin treated loops was less than controls which can be interpreted as due to a preferential increase in intracellular water. Inulin is employed as a marker which is considered to distribute solely to the extracellular space of most tissues. The distribution of inulin in the present study is too great to be considered an accurate measurement of the extracellular space, since these spaces exceed the acceptable values for the extracellular space of intestinal tissue (18). Significant amounts of inulin may distribute to the intracellular space in intestinal mucosa as well as to a larger extracellular space than dextran since inulin molecular size is considerably smaller. Also, inulin has unusual solubility properties, being quite insoluble in aqueous solutions. Its solubility may decrease in the presence of the polysaccharides of the interstitial ground substance and subsequent precipitation would give higher values for the extracellular space. Clearly, no conclusions can be made on what space inulin measures in this tissue. The amounts of inulin and dextran which are present in loop fluid are small which suggests that permeability of at least the luminal mucosal border is not increased by cholera toxin.

The albumin space was significantly increased by cholera toxin while the red blood cell space increased, but not significantly which indicates that the extravascular albumin space increased. Part of the increase in the albumin space may be said to be due to the small increase in the intravascular space even though the latter increase was not significant. The following calculation shows that the increase in the extravascular albumin space is still significant even if the increase in the intravascular space were significant. Assuming that the hematocrit of intestinal blood is 20 to 25% (16), calculations based on the increased red cell volume in the toxin treated loops would suggest an increase of approximately 11 μl/g tissue to the reported normal intravascular albumin space of about 39 μl/g tissue (16). If the former value is added to the control albumin space

TABLE V. Urea-^{14}C Space (5 hr).

Loop section[a]	Expt.	Urea space (μl/g)	HCT (%)	Tissue H_2O (%)
Proximal	Control[b]	748 ± 16[c]	48 ± 1	76.0 ± 0.5
	Toxin[d]	754 ± 6	54 ± 1.5[e]	78.6 ± 0.7[e]
Distal	Control[b]	730 ± 21	48 ± 1	74.9 ± 0.5
	Control[d]	729 ± 26	54 ± 1.5[e]	75.1 ± 0.6

[a] $N = 6$ for each group.
[b] One proximal and one distal loop from same animal.
[c] Mean ± SEM; μl/g wet tissue wt.
[d] One proximal and one distal loop from same animal.
[e] Significantly different from controls $p < .05$.

the difference between toxin and control tissues is still significant ($p < .05$). Thus, the increase in this space is largely due to a significant increase in the extravascular distribution of serum albumin.

Protein bound ^{125}I was found in the loop fluid. This cannot be assumed to indicate serum albumin movement across the mucosal cells since RISA-125 entering the epithelial cell could be catabolized and deiodinated, and free ^{125}I could move into the intestinal lumen and bind rapidly to mucous proteins.

Both albumin and dextran distribute to the same compartments, yet cholera toxin increased the space of the former while that of the latter did not change. This extravascular, extracellular compartment consists of a structured phase composed of fibroblasts, fibers (collagen, reticular and elastic), and ground substance (aggregated glycosaminoglycans and glycoproteins). This hydrated colloid-rich phase is in equilibrium with an essentially unstructured water-rich phase, which contains soluble colloidal components and is in a steady state equilibrium with the water and electrolytes of blood and cells. Serum albumin should distribute to the water-rich phase but it is excluded from the structured colloid-rich phase. The degree of exclusion is dependent on the concentration of polysaccharides in the phase, and the phenomenon of exclusion illustrates the steric exclusion of a macromolecule by randomly coiled polysaccharides (19). Both morphological and kinetic studies show that intestinal capillaries are very permeable to large molecules (15, 16) suggesting that they do not determine the extravascular volume of distribution of serum albumin. The limits of serum albumin distribution may well be determined by the structured phase (19). Depolymerization or disaggregation of this phase would decrease the excluded volume for albumin and hence increase its space in the tissue. The excluded volume for a molecule is proportional to its radius and its degree of assymetry (19). These physical properties of serum albumin (mol wt 69,000) are greater than those of dextran (mol wt 60,000–90,000). This could explain the difference between the albumin and dextran space. Also, if the excluded volume for dextran is normally small, the dextran space would increase little following disaggregation of the structured phase.

Disaggregation of the structured phase would increase the colloid osmotic pressure of fluid in the interstitial space. This would result from an increase in the amount of serum albumin and from the increase in number of particles following disaggregation of the polymers. It has been shown that disaggregation of the structured phase results in a space holding more water, possibly progressing to edema (20). An increased interstitial colloid osmotic pressure would blunt or nullify the plasma colloid osmotic force acting across the capillary membrane. It has been proposed that dilution and washout of extravascular proteins represent a control mechanism which limits filtration of capillary fluid and aids in its return (21, 22). This component of fluid homeostasis would be absent in the cholera toxin treated tissues. With the approach of a balance between plasma and tissue colloid osmotic pressure, the only forces left to determine the direction of fluid fluxes are capillary and interstitial pressures. Very small hydrostatic pressures on the capillary side of intestinal epithelial cells are required for net secretion of fluid to result (23). Thus, capillary hydrostatic pressure could become the principal driving force for fluid production in cholera.

Summary. The addition of cholera toxin to closed loops of proximal small intestine resulted in an increase in the extravascular space of serum albumin. It did not alter the tissue red blood cell, dextran or urea spaces while causing a decrease in the inulin space. The increase in the albumin space suggests that the interstitial colloid osmotic pressure was elevated.

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