Significance of Abnormal Rabbit Ileal Histology in the Pathogenesis of Diarrhea

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Received for publication 31 July 1979

In spite of several macroscopic criteria for predicting the presence of histological abnormalities in rabbit ileum, microscopic ileal abnormalities still can escape detection. The effect of histologically abnormal rabbit ileum was evaluated on basal intestinal absorption, on basal absorption, on basal adenylate cyclase activity, and on cholera toxin-induced secretion and cholera toxin-induced stimulation of adenylate cyclase activity. Compared to histologically normal rabbit ileum, the presence of histological abnormalities was associated with decreased basal intestinal water, Na, Cl, and glucose absorption, absent glucose-dependent water absorption, and elevated basal adenylate cyclase activities. However, histologically abnormal rabbit ileum responded to inoculation of purified cholera toxin with stimulation of intestinal water secretion and adenylate cyclase activity similar to that in histologically normal ileum. These data have implications concerning the design of experiments that attempt to study the pathogenesis of diarrheal diseases by correlating changes in ileal transport with changes in ileal mucosal adenylate cyclase activity. In spite of abnormal ileal histology, studies of intestinal secretory states which attempt to define the role of adenylate cyclase in secretory processes can be performed provided animals are used as their own controls. However, when groups of animals are compared, the presence of an histologically abnormal ileum can cause changes in basal and intestinal secretagogue-stimulated ileal water and electrolyte transport and in basal and intestinal secretagogue-stimulated mucosal adenylate cyclase activity which can lead to erroneous conclusions if the presence of the abnormal ileal histology is not considered.

Much of the information on the pathogenesis of diarrheal illnesses has resulted from studies in which the effects of intestinal secretagogues on intestinal transport have been correlated with the effects of the same secretagogues on the activities of intestinal enzymes that are thought to be intracellular mediators of intestinal mucosal absorptive and secretory processes. In this way, increased mucosal adenylate cyclase activity has been associated with intestinal water and electrolyte secretion (7, 10, 14, 17), and altered intestinal Na-K-adenosine triphosphatase activity has been associated with increased (2, 4) and decreased absorptive function (3). The most commonly used animal model in studies of both normal and abnormal intestinal water and elec-

trolyte transport has been the rabbit ileum. Active electrolyte transport has been extensively characterized in this tissue in vitro (8, 15, 16), and more recently, water and electrolyte transport have been well characterized in vivo (5). Among the difficulties in using rabbit ileum for intestinal transport studies have been the frequent infestation of rabbit ileum by several pathogenic organisms including the protozoa coccidia (1) and the presence of rabbits which demonstrate abnormal active Cl transport in vitro under basal conditions (13; Y. H. Tai, R. A. Decker, M. Donowitz, A. N. Charney, and J. A. Wright, *Fed. Proc.* 37:513, 1978).

In the most detailed evaluation of the effects of endemic intestinal disease, Al-Awqati et al.

(1) demonstrated that abnormal ileal histology (both related to and independent of coccidiosis) was associated with (i) decreased active net Na and Cl absorption to values not significantly different from zero; (ii) increased tissue electrical resistance; and (iii) a decreased short-circuit current response to glucose. In contrast to this decrease in absorptive function, abnormal ileal histology was characterized by a normal short-circuit current response to theophylline. Previous attempts to identify animals with abnormal histology without actually obtaining microscopic histology have relied on the presence of (i) clinical diarrhea manifest by perineal staining; (ii) a large amount of ascites; (iii) gross serosal thickening; (iv) a large volume of intraluminal fluid; (v) failure to respond to glucose in vitro with a significant increase in short-circuit current; and (vi) possession of a high electrical tissue resistance (1; Tai et al., *Fed. Proc.* 37:513, 1978). However, it has not been demonstrated whether histologically abnormal ileal mucosa can always be detected by applying these criteria. Furthermore, secretion of water and electrolytes has been demonstrated in rabbit ileum in vivo in the absence of the above criteria of abnormality (5, 9).

The current study was designed to determine whether the wide range of water and electrolyte transport values measured in rabbit ileum in vivo is attributable to the presence of histological abnormalities, and whether histological examination of ileal tissue is necessary to rule out functionally significant mucosal lesions. In addition, an attempt was made to determine whether ileal histological abnormalities are accompanied by characteristic alterations in mucosal adenylate cyclase activity and, using cholera toxin as an example of an intestinal secretagogue, to determine whether histological abnormalities alter the ability to detect intestinal secretagogue-induced increases in adenylate cyclase activity.

MATERIALS AND METHODS

The study populations were obtained retrospectively from studies of three separate litters of male albino New Zealand rabbits obtained from a single animal supplier, and the experimental groups were separated strictly on the basis of whether the ileal histology was normal or abnormal when coded slides were reviewed. Animals with normal and abnormal histology were present in each litter. All rabbits weighed 2 to 2.5 kg and were maintained on a standard rabbit chow diet with free access to water. As far as could be determined, none of the animals had received antibiotics. None of the animals studied had perineal staining, large amounts of ascitic fluid, serosal thick-

ening, or an increased amount of liquid stool within the ileal lumen.

Transport studies. Each animal was lightly anesthetized with sodium pentobarbital (30 mg/kg), and three 15-cm ileal loops were constructed, separated from each other by a 5-cm blank space. Ileal loops began 70 cm and ended 15 cm proximal to the ileo-appendiceal mesenteric attachment. The loops were washed with warm saline, and the proximal loop was inoculated with 1 ml of 0.9% saline. The middle loop was inoculated with 1 ml of saline containing 50 μ g of purified cholera toxin (cholera toxin; Schwarz-Mann, Orangeburg, N.Y.). The most distal loop was not inoculated and was cannulated at both ends. All three loops were returned to the abdomen.

Net ileal transport of water, Na, Cl, and glucose was measured in the distal ileal loop by a modification of the in vivo perfusion technique we have previously described (2, 5). The loops were perfused at a constant temperature (37°C) and rate (0.5 ml/min) with a peristaltic pump (model 1203; Harvard Apparatus Co., Millis, Mass.). Body temperature was maintained at 37°C with a thermocouple-controlled heating lamp. The perfusion study consisted of an initial 150 min during which the animals were perfused with a Ringer's HCO_3 solution (composition, in millimoles per liter: Na, 140; K, 5.2; Cl, 119.8; HCO_3 , 25; Ca, 1.2; Mg, 1.2; HPO_4 , 2.4; H_2PO_4 , 0.4) equilibrated with 95% O_2 -5% CO_2 containing 15 mM mannitol, polyethylene glycol of molecular weight 4,000 (5 g/liter), and ^{14}C -polyethylene glycol as a nonabsorbable water marker (osmolarity 295 mosmol/kg, pH 7.4). The perfusion consisted of a 90-min steady state followed by three 20-min collection periods. In most animals this perfusion was followed by another 150-min perfusion in which the ileal loop was perfused with an identical perfusate, except that the mannitol was replaced with 15 mM glucose. This perfusion also consisted of a 90-min steady state followed by three 20-min collection periods. After the perfusion studies, the length of each loop was measured in a uniform manner and immediately prepared for adenylate cyclase determination. In preliminary studies, the order of perfusion with glucose or mannitol did not alter either the subsequent perfusion study or the mucosal adenylate cyclase activity.

Net water, Na, Cl, and glucose transport were calculated as previously described (2) and expressed as microliters, microequivalents, or micromoles per hour per centimeter length of perfused ileal segment. Net absorption from the lumen was expressed as a positive value; net secretion into the lumen was expressed as a negative value. Mean values were obtained by averaging the results of the three collection periods. For each perfusion period, polyethylene glycol recovery was determined; periods in which polyethylene glycol recovery was not $100 \pm 6\%$ were discarded. ^{14}C -polyethylene glycol activity was measured in a Beckman liquid scintillation system (LS-345; Beckman Instruments, Inc., Fullerton, Calif.). Quench corrections were made by the method of external standards. Sodium, Cl, and glucose were measured by the flame photometry, coulometric titration, and ortho-toluidine methods, respectively.

Five hours after 0.9% saline and cholera toxin inoculation, the volume of loop contents and loop lengths were determined, and mucosal adenylate cyclase activity was then measured.

After the perfusion and measurement of loop contents and lengths, tissue was sampled for histology from the center of the loop as well as just proximal to each loop. The tissue was oriented with the villi up and placed in 10% Formalin, stained with hematoxylin and eosin and alcian blue-periodic acid-Schiff, coded, and examined by one of the authors. The tissues were separated into those that were histologically normal or abnormal, based on whether there was blunting of the villi, hyperplasia of the crypts, or epithelial surface abnormalities consisting of conversion from columnar to cuboidal cells. Although in the abnormal ileal tissue there was also an increase in the mononuclear infiltrate in the lamina propria and an increase in the intraepithelial lymphocytes, tissue was not categorized as abnormal on this basis. When present, microscopic histological abnormalities were present in all ileal segments, although the severity often varied. Neither inoculation of 0.9% saline or cholera toxin nor intestinal perfusion appeared to cause any change in histology as compared to ileal mucosa sampled proximal to each loop. As previously described, cholera toxin inoculation appeared to decrease goblet cell mucus (6).

Adenylate cyclase activity. Mucosa was obtained from the perfused and inoculated intestinal segments by scraping with a glass slide. The mucosa was homogenized with an iced sintered glass homogenizer in a solution containing 75 mM tris(hydroxymethyl)aminomethane and 25 mM $MgCl_2$ (pH 7.6). The whole homogenate was assayed for adenylate cyclase by the method of Krishna et al. (11) with minor modifications as previously described (2, 4). All samples were assayed within 1 min of removal of the intestinal segment from the animal, and all animals were alive at the time the intestinal segment was removed. Approximately 50 μ g of homogenate protein was incubated for 10 min at 37°C in a solution containing 1.5 mM adenosine triphosphate, 1 μ Ci of (α - ^{32}P)adenosine triphosphate (New England Nuclear Corp., Boston, Mass.), 10 mM $MgCl_2$, 10 mM theophylline, 20 mM tris(hydroxymethyl)aminomethane, 5 mM phosphoenolpyruvate, 50 μ g of pyruvate kinase per ml, and 20 μ g of myokinase per ml. After passage over columns of Bio-Rad (AG50W-X4) resin (BioRad Laboratories, Richmond, Calif.) and alumina, the radioactive cyclic adenosine 3',5'-monophosphate in the eluate was measured in a Beckman beta scintillation counter. Appropriate corrections were made for incubations run without enzyme and for the incomplete recovery of cyclic adenosine monophosphate. Results were expressed as picomoles of cyclic adenosine monophosphate formed per milligram of protein per 10 min.

Protein concentrations were determined by the method of Lowry et al., using standards of rabbit serum albumin (12).

Statistical analyses were performed by Student's *t* tests for paired or unpaired data and were two-tailed; linear regression analysis was performed by the method of least squares (18). All results were expressed as mean $\pm$ standard error.

RESULTS

Division of the animals into the two study groups was done on the basis of ileal histology. Compared to the histology of normal ileum, a wide range of structural abnormalities was observed (Fig. 1). In addition to blunting of intestinal villi, there were severe surface epithelial cell abnormalities with a predominance of cuboidal epithelial cells and increased lymphocytic infiltration. No gross or microscopic ulcerations were seen. There was crypt cell hyperplasia and an increase in the infiltration of the lamina propria with plasma and lymphoid cells. Although coccidia were not identified in spite of multiple sections, histological abnormalities similar to those seen in the present study have been described in animals in which coccidia also were identified (1). This parasite could have been present but not observed. Although the severity of the histological abnormalities varied among the segments sampled for histology in individual animals, all animals could easily be classified as having either normal or abnormal histology.

Association of ileal histological abnormalities with abnormalities in transport. In rabbits with histologically normal ilea, the levels of water (Table 1), Na (18.20 ± 3.40 μ Eq/h per cm), and Cl absorption (21.50 ± 4.80 μ Eq/h per cm) from the Ringer's HCO_3 -mannitol perfusate were similar to levels previously reported from this laboratory (5). In addition, after perfusion of the solution containing 15 mM glucose, glucose absorption was 7.8 ± 0.9 μ mol/h per cm and glucose-dependent water absorption was 67.8 ± 28.7 μ Eq/h per cm. In contrast, water and electrolyte absorption was significantly reduced in histologically abnormal ilea. Net water, Na (-0.12 ± 1.08 μ Eq/h per cm), and Cl (-1.05 ± 2.90 μ Eq/h per cm) transport was markedly abnormal and not significantly different from zero in animals with histologically abnormal ilea (Table 1). In addition, glucose absorption was significantly decreased in animals with histological abnormalities, and there was no significant glucose-dependent water absorption observed. However, there was no correlation between the severity of the histological abnormalities with the quantitative abnormalities in intestinal transport.

Although histological abnormalities were associated with changes in basal ileal transport, the effect of histological abnormalities on cholera toxin-induced ileal secretion was less clear. The volume/length ratio in cholera toxin-inoculated ileal loops was similar in animals with and without ileal histological abnormalities (Table 1). This suggests that stimulation of ileal

FIG. 1. (A) Abnormal ileum: blunted villi; cuboidal epithelial cells; crypt hyperplasia; increased cellular infiltrate in lamina propria including polymorphonuclear cells. (B) Abnormal ileum: blunted villi; cuboidal epithelial cells; decreased total mucosal thickness; increased cellular infiltrate in lamina propria including polymorphonuclear cells; increased goblet cells.

secretion is possible in the presence of histologically abnormal ileum. Because of the insensitivity of the technique used, however, it is unclear whether abnormal ileal histology alters the magnitude of the secretory response.

Association of ileal histological abnormalities with altered adenylate cyclase activity. After the intestinal perfusion and inoculation studies, ileal mucosal adenylate cyclase activity, measured in animals with normal histology, was 390 ± 33 pmol of cyclic adenosine monophosphate per mg of protein per 10 min (Table 2). This level of rabbit ileal adenylate cyclase activity is similar to results previously reported from this laboratory (5). In contrast, in animals with histologically abnormal ilea, adenylate cyclase activity is similar to results previously reported from this laboratory (5). In contrast, in animals with histologically abnormal ilea, adenylate cyclase activity was significantly increased. There was no overlap in adenylate cyclase activities in animals with normal and those with abnormal ileal histology (Fig. 2). Of note was that when all animals were considered, and when groups with normal and abnormal ileal histology were analyzed separately, there

was no significant correlation between the level of adenylate cyclase activity and net water (Fig. 1), Na, or Cl transport.

Five hours after inoculation of cholera toxin, ileal adenylate cyclase activity had increased by 423 ± 94 pmol of cyclic adenosine monophosphate per mg of protein per 10 min in histologically normal ileum (Table 2). Although adenylate cyclase activity after cholera toxin inoculation was significantly greater in histologically abnormal rabbit ileum, cholera toxin increased ileal adenylate cyclase activity by a similar absolute amount. Thus, the cholera toxin-induced increase in adenylate cyclase activity was similar regardless of the ileal histology.

DISCUSSION

These studies demonstrate that histologically abnormal rabbit ileum behaves differently from histologically normal ileum in both basal absorptive function and basal adenylate cyclase activity. Specifically, compared to histologically normal ileum, histologically abnormal tissue has decreased basal absorption of water, Na, Cl, and glucose, absent glucose-dependent water absorption, and increased basal adenylate cyclase ac-

TABLE 1. Association of abnormal histology and ileal transport^a

Histology	N	Net water movement (μ liters/h per cm)	Glucose absorption (μ mol/h per cm)	Glucose-dependent water absorption (μ liters/h per cm)	Volume/length (ml/cm) in:		P ^b
					Saline-inoculated loop	Cholera toxin-inoculated loop	
Normal	7	97.5 ± 10.2	7.8 ± 0.9	67.8 ± 28.7	0.12 ± 0.10	1.36 ± 0.39	<0.01
Abnormal	6	-0.6 ± 5.4	3.3 ± 1.2	24.0 ± 20.1	0.21 ± 0.25	0.91 ± 0.20	<0.05
P ^c		<0.001	<0.002	NS	NS	NS	

^a Results are mean $\pm$ standard error. N represents number of animals studied. Glucose absorption was determined in five animals with normal histology and five with abnormal histology. P^c values represent comparisons between animals with normal and abnormal ileal histology (unpaired *t* test). P^b values represent comparisons between cholera toxin-inoculated and saline-inoculated loops in individual animals (paired *t* test). NS, Not significant.

TABLE 2. Association of abnormal histology and ileal adenylate cyclase activity^a

Histology	N	Adenylate cyclase activity (pmol of cAMP per mg of protein per 10 min)		Cholera toxin-induced increase in activity	P ^{**}
		Saline-inoculated loop	Cholera toxin-inoculated loop		
Normal	7	390 ± 33	813 ± 63	423 ± 94	<0.005
Abnormal	6	710 ± 68	$1,112 \pm 78$	402 ± 140	<0.02
P [*]		<0.005	<0.02	NS	

^a Results are mean $\pm$ standard error; activity is expressed as picomoles of cyclic adenosine monophosphate per milligram of protein per 10 min. N represents number of animals studied. P^{*} values represent comparisons between animals with normal and abnormal ileal histology (unpaired *t* test); P^{**} values represent comparisons between cholera toxin-inoculated and saline-inoculated loops in individual animals (paired *t* test). NS, Not significant.

FIG. 2. Relationship between ileal adenylate cyclase activity and net water movement in animals with normal and abnormal ileal histology. There is no significant relationship between the level of ileal adenylate cyclase activity and net water movement either when all animals are considered or when the animals with normal and abnormal histology are considered separately.

tivity. Since histologically abnormal tissue often appears normal on gross examination and may not be accompanied by perineal staining, a large amount of ascites, gross serosal thickening, or a large volume of intraluminal fluid, it is necessary to obtain intestinal histology to identify animals with abnormal small-intestinal histology. It is worth speculating whether the wide variation in basal water and electrolyte transport values and adenylate cyclase activities reported in studies of rabbit ileum is partially accounted for by the presence in the studies of animals with both normal and abnormal histology.

It is equally important that, in spite of abnormal ileal histology and elevated levels of basal mucosal adenylate cyclase activity, inoculation of cholera toxin resulted in a level of fluid secretion and in an increase in adenylate cyclase activity that were quantitatively similar to that induced by cholera toxin in histologically normal tissue. Al-Awqati et al. also demonstrated that the active electrolyte secretory response to theophylline *in vitro*, as determined by the increment in short-circuit current, was normal in histologically abnormal rabbit ileum (1).

The current study has implications concerning the design of experiments that attempt to define the pathogenesis of diarrheal diseases by correlating alterations in the intestinal water and electrolyte transport with alterations in mucosal adenylate cyclase activity. Studies of intestinal secretory states or the role of the enzyme adenylate cyclase in a secretory process can be performed in spite of abnormal ileal histology as

long as animals are used as their own controls. In contrast, only animals with histologically normal ilea can be used in studies that compare intestinal transport and mucosal adenylate cyclase activity between groups of animals. The current results suggest that studies which attempt to measure ileal water and electrolyte transport, mucosal adenylate cyclase activity, or both, without considering the ileal histology may be misleading.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AM-20700 from the National Institute of Arthritis, Metabolism and Digestive Diseases by the Medical Research Service of the Veterans Administration, and by a grant from The Medical Foundation, Boston, Mass. M. Donowitz is a recipient of Public Health Service Research Career Development Award K04-00588 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

We gratefully acknowledge the assistance of Jerry Trier in establishing the histological criteria used in this study.

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