Human Rotavirus Enteritis Induced in Conventional Piglets

INTESTINAL STRUCTURE AND TRANSPORT

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ABSTRACT To better understand the pathogenesis of infantile viral gastroenteritis, we studied Na^+ and Cl^- fluxes in vitro in short-circuited jejunal epithelium from 8–10-day-old piglets after infection with a standard dose of human rotavirus given via nasogastric tube. 11 infected piglets, all of whom became ill, were compared with 9 uninfected, healthy litter-mates. When killed 72 h after infection, intestinal villi were shorter and crypts deeper ($P < 0.025$) in duodenum, upper jejunum, and mid-small intestine, but not ileum in infected piglets. Virus antigen was seen by fluorescence microscopy in occasional jejunal villus tip cells in only four infected piglets and no controls at 72 h. Net Na^+ and Cl^- fluxes did not differ from noninfected litter-mate controls under basal conditions, but response to glucose was blunted in infected piglets ($P < 0.001$). Theophylline stimulated net Cl^- secretion in both infected and control animals, and cyclic AMP concentration in isolated jejunal villus enterocytes did not differ significantly. In isolated jejunal villus enterocytes of infected piglets, thymidine kinase activity increased ($P < 0.001$), and sucrase activity decreased ($P < 0.001$). We conclude that in this invasive enteritis caused by a major human viral pathogen, glucose-coupled Na^+ transport is impaired in the jejunum at a time when the villus epithelium shows enzyme characteristics of crypt epithelium, and when little or no virus is present. These findings are identical to those occurring in an invasive coronavirus enteritis of piglets but differ markedly from those seen with enterotoxigenic diarrhea.

INTRODUCTION

A specific virus, initially observed in duodenal mucosa of infants and children with acute gastroenteritis (1, 2) is now recognized as a major cause of acute infectious diarrhea in this age group throughout the world (3). In developed countries, 40–50% of all cases of infectious diarrhea admitted to hospital are caused by this virus; the incidence may rise to 80% in the cooler months of the year (4–7). Although it possesses specific morphologic, antigenic, and biochemical characteristics, the virus is not yet fully characterized. As a result, many names have been assigned to it, including orbivirus (2), duovirus (4), rotavirus (8), reovirus-like agent (9), and infantile gastroenteritis virus (10). Human rotavirus (HRV)[1] now seems to be the name most favored. Inasmuch as preliminary experiments suggested that conventional and gnotobiotic piglets are susceptible to HRV infection (11), we administered the virus to young, conventionally-reared piglets and studied intestinal structure and function at the height of the ensuing illness. We measured Na^+ and Cl^- trans-

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[1] *Abbreviations used in this paper*: cAMP, cyclic AMP; HRV, human rotavirus; ISc, short-circuit current; PD, potential difference.

port in jejunal epithelium because clinical experience indicates that abnormal ion transport plays a major role in the pathogenesis of the acute diarrhea seen in infants with HRV enteritis (12). Furthermore, in transmissible gastroenteritis, a different specific invasive viral enteritis occurring in young pigs, a specific defect in sodium transport is a major factor in the pathogenesis of diarrhea (13–16).

METHODS

8–10-day-old piglets from five litters of conventional York swine weaned at 5 days, were studied after a 4-ml inoculum, containing human rotavirus particles ($\cong 10^8$ per ml), was given by nasogastric tube. The inoculum, prepared as an ultrafiltrate (0.45 nm Millipore, Millipore Corp., Bedford, Mass.) of stool from infected infants, was not concentrated. Infected piglets were isolated in special facilities. Controls were age-matched uninfected litter-mates. A similar dietary intake was maintained in both groups. No sow in the herd selected for study had antibodies to human rotavirus. All infected animals were killed by intracardiac pentobarbital Na injected 72 h after receiving the virus; preliminary studies had shown that diarrhea was most severe by that time. Confirmation of HRV infection was made by electron microscopic identification of virus particles in intestinal juice or by fluorescent microscopy of small intestinal mucosa at post mortem.

Immediately after death a 65-cm segment of jejunum starting 5 cm distal to the ligament of Treitz was removed from each animal: the upper 15 cm and lower 30 cm for isolation of villus enterocytes, 2 cm for histological and immunofluorescence studies, 5 cm for analysis of mucosal enzymes, and 13 cm for short-circuited chamber studies. Segments of ileum, starting 10 cm proximal to the ileocecal junction and mid-small intestine, were also removed: 30 cm for isolation of villus enterocytes, 2 cm for histological and immunofluorescence studies, and from ileum 5 cm for mucosal enzyme analyses. A 7-cm segment of duodenum, starting 10 cm distal to the pylorus was taken for histological, immunofluorescence, and mucosal enzyme analyses. Segments of stomach and proximal colon were also taken for histological and immunofluorescence studies. Samples of small intestinal and colonic contents were taken for bacterial culture and virus identification by negative contrast stain electron microscopy.

Tissue for light microscopy was fixed in Bouin's reagent, blocked in paraffin, and stained with hematoxylin and eosin. All sections were examined without prior identification by the same person who used a calibrated micrometer eye piece to measure villus height and crypt depth in three adjacent properly-oriented representative villi from each section. Epithelial injury was semiquantified (16). Sections for fluorescence microscopy were fixed in 95% ethanol at 4°C (17). Sera, previously shown to be highly specific for HRV (18), obtained from guinea pigs after inoculation with human rotavirus, were used as the antibody and pre-inoculation sera as control. Fluorescein-labeled rabbit immunoglobulin to guinea pig gamma globulin was the marker. Slides were mounted using Elvanol (19) (E. I. du Pont de Nemours & Co., Wilmington, Del.), and specificity was checked by preliminary incubation of purified human rotavirus with control and antibody-positive sera (17).

To measure ion flux, jejunal mucosa was stripped of muscularis, and four adjacent portions were mounted in Ussing chambers (15). The tissues were bathed with Krebs bicarbonate buffer, and $^{22}Na^+$ and $^{36}Cl^-$ were added to the mucosal or serosal side (16). Unidirectional and net Na^+ and Cl^- fluxes were measured in the absence of electrical or chemical gradients. The spontaneous mucosal potential difference (PD) was neutralized by manually introducing an appropriate short-circuit current (ISc) (20, 21). Tissue was studied for three consecutive 70-min periods; during the first, Na^+ and Cl^- fluxes were measured under basal conditions, without glucose; for the second, 30 mM glucose was added to both sides of the tissue; and for the third, 10 mM theophylline was added to both sides. During each period, the tissue was allowed to equilibrate for 40 min and then 1 ml-samples were withdrawn from the bathing solutions at 10-min intervals for radioisotope counting; three consecutive 10-min fluxes and one overall 30-min flux were calculated. Unidirectional fluxes were derived from a standard formula (22); net fluxes were calculated from a comparison of the unidirectional fluxes from mucosa to serosa and from serosa to mucosa of paired strips of tissue. Conductance (mmho 0.01 cm) was calculated from PD and ISc readings measured at 10-min intervals. Homogenates of mucosa scraped from 5-cm segments of intestine with a glass slide were used to assay total sucrase, maltase, lactase, and Na^+K^+ATPase activities by conventional techniques (13), and the data were related to the protein content of the tissue (13) in the duodenum, upper jejunum, and ileum. Also, sucrase and thymidine kinase activities were measured in enterocytes selectively isolated by a vibration technique from villi of intestinal segments of upper jejunum, mid-small intestine, and ileum (23). The tissue remaining after villus cell isolation was checked by microscopy for the presence of intact crypts (23).

Cyclic AMP (cAMP) was measured using the method of Gilman (24) in isolated upper jejunal villus enterocytes after treatment with trichloracetic acid to a final concentration of 5% and purification on a Dowex 50-H^+ column (25) (Dow Chemical Co., Midland, Mich.).

Results were analysed using Student's t test or where appropriate the paired t test. Because enzyme activities follow a log normal skew distribution, all enzyme data were converted to logarithms before analysis and are expressed as antilogarithms.

RESULTS

Clinical, microscopic, and microbiologic findings. All pigs given human rotavirus became ill; 7 of 11 developed diarrhea 48–68 h after infection, and the remainder had fluid distention of the small intestine and liquid cecal contents at post mortem. All control animals were healthy. Serum electrolyte and acid base studies did not differ between study groups. Electron microscopy of small intestinal or colonic content was positive for rotavirus in all infected piglets but in no controls. Bacterial cultures of gut contents collected at post mortem were negative for pathogenic enteric organisms in all animals.

On light microscopy a lesion of the intestinal mucosa was seen in all infected animals. In the duodenum, upper jejunum, and mid-small intestine, but not the ileum of all infected animals, there was significant shortening of the villi and deepening of crypts (Table I). For example, in the jejunum the

TABLE I

Measurements of Small Intestine Mucosal Structure in Control and Infected Piglets*

	Duodenum		Upper jejunum		Mid-small intestine		Ileum	
	n		*n*		*n*		*n*	
Villus height, μm								
Control	7	405.0±25.6	7	378.0±18.9	8	388.1±32.5	8	291.8±18.8
Infected	7	178.3±28.2	7	160.4±25.7	8	198.4±35.4	8	240.0±21.9
P		<0.005		<0.001		<0.010		NS
Crypt depth, μm								
Control	7	170.0±9.0	7	144.0±6.0	8	125.3±6.5	8	121.5±11.0
Infected	7	257.1±19.0	7	244.2±24.6	8	180.8±17.7	8	165.8±17.3
P		<0.010		<0.010		<0.025		NS

* Mean±1 SE.

mean villus/crypt ratio was 2.63 in controls and 0.66 in infected animals. Epithelial cell morphology showed marked abnormalities in tissue from infected animals with cuboidal cells, centrally placed nuclei, and poorly defined brush borders (Fig. 1). Sections from controls were normal. Sections of stomach and colon were normal in both study groups.

Fluorescence microscopy showed patchy particulate supranuclear fluorescence in enterocytes on the tips of occasional villi in the duodenum and upper jejunum and mid-small intestine in four infected animals and in no controls. Of the four with positive findings, three did not have diarrhea at the time of post mortem; the fourth piglet developed diarrhea shortly before death. Specific fluorescence was not found in the mucosa of stomach, ileum, or colon of any animal.

Short-circuited chamber studies. (*a*) Na^+ flux (Table II). Under basal conditions, without glucose, unidirectional fluxes were significantly less in tissue from HRV-infected animals, but net fluxes were secretory and did not differ significantly from controls. After the addition of 30 mM glucose, control tissue responded with a significant increase in net Na^+ flux ($P < 0.001$). In tissue from infected piglets the response of net Na^+ glux to glucose was blunted and significantly different from controls ($P < 0.001$). When 10 mM theophylline was added after glucose, the change in net Na^+ flux in control tissue, from 2.10±0.50 to 0.36±0.77 did not differ significantly from the change seen in infected tissue, from 0.98 ±0.46 to −2.34±0.40 $\mu eq/cm^2$ per hour.

(*b*) Cl^- flux (Table II). Under basal conditions net Cl^- fluxes in infected animals, like net Na^+ fluxes, were secretory and not significantly different from controls; they remained secretory in both controls and infected piglets when 30 mM glucose was added. Net Cl^- fluxes were significantly decreased in both control and infected groups (−2.40 vs. −3.04 $\mu eq/cm^2$ per hour) when theophylline was added. Unidirectional Cl^- fluxes in infected pigs were less than controls under all study conditions.

(*c*) Electrical data (Fig. 2). Under basal conditions, mean PD in tissue from infected animals was significantly higher than in controls ($P < 0.001$); ISc did not differ significantly between study groups. Addition of 30 mM glucose significantly increased PD and ISc in both groups ($P < 0.001$), but the increments were significantly less ($P < 0.01$) in tissue from infected animals. Theophylline significantly increased ISc in both groups ($P < 0.001$) and PD in tissue from HRV-infected piglets ($P < 0.001$), but not in control tissue. Conductance of tissue from infected piglets was significantly lower than control tissue under all experimental conditions; theophylline significantly increased conductance in tissue from controls ($P < 0.001$) and significantly decreased it in tissue from infected animals ($P < 0.001$).

Enzyme and cAMP analyses. In mucosal homogenates from HRV-infected piglets, activities of sucrase, lactase, maltase, and Na^+K^+ATPase were significantly decreased in the upper jejunum; sucrase, lactase and Na^+K^+ATPase in the duodenum and lactase alone were decreased in the ileum (Table III). After infection thymidine kinase activity in epithelial cells isolated specifically from villi of upper jejunum increased fivefold (3.2 vs. 17.1, $P < 0.001$), and sucrase activity decreased 10-fold (72.5 vs. 7.3, $P < 0.001$) (Fig. 3). A similar pattern with significant differences between study groups occurred in the mid-small intestine but not in the ileum (Fig. 3).

Cellular cAMP levels measured in enterocytes isolated from villi of the upper jejunum were similar in control and infected piglets (control 10.1±3.9, infected 11.8±6.9 pmol/mg protein).

DISCUSSION

Our findings in young pigs given HRV resemble those seen in the human disease: viral invasion of the villus epithelium (2), a lesion of the jejunal

mucosa (2), depressed activities of mucosal disaccharidases (2), and watery diarrhea (12). All animals in the infected group were diseased, but perhaps not all were at the same stage of infection when studied 72 h after they had received the virus. The variations in symptoms in our animals accord with the reported variability of the response of piglets to HRV infection (11, 26, 27). In part, these apparent variations may be due to the variable distribution and extent of the infection in the gut. From human studies it is clear that HRV invasion of the mucosa can be patchy (2, 5).

Despite clinical variations in this animal model, specific segments of bowel removed at a fixed time after infection showed highly significant abnormalities in mucosal structure and ion transport when compared with tissue from uninfected controls. Villi were shortened, and therefore, the absorptive surface of mature epithelium diminished in the upper bowel, the region known to be the target preferred by HRV (1, 2). These light microscopic data are supported by the diminution in infected pigs of certain enzyme activities normally found in mature villus epithelium; disaccharidases and Na^+K^+ATPase for example. Decreased absorptive surface area may contribute to intestinal malfunction in HRV enteritis, but defective active ion flux is likely to be of even greater importance in the pathogenesis of the acute diarrheal state. In short-circuited jejunal epithelial membranes, the effects of glucose on sodium and chloride transport and on electrical phenomena were severely blunted. These abnormalities undoubtedly contribute to the disturbed electrolyte absorption that appears to be a major factor in the pathogenesis of HRV diarrhea. Under basal conditions, jejunum from infected pigs secreted, but so did controls. Net secretion, a constant finding in short-circuited jejunum from normal piglets studied under basal conditions has been observed also in rabbits (28). We suspect that our in vitro data, with rather than without glucose, reflect in vivo conditions in the jejunum of young pigs. Diminished activity of the Na^+K^+ATPase system implies defective active extrusion of sodium at the lateral membrane of the enterocyte, a defect that could contribute further to defective sodium transport. Diminished disaccharidase activities in the proximal and mid segments of the small bowel suggest a potential role for sugar malabsorption in HRV diar-

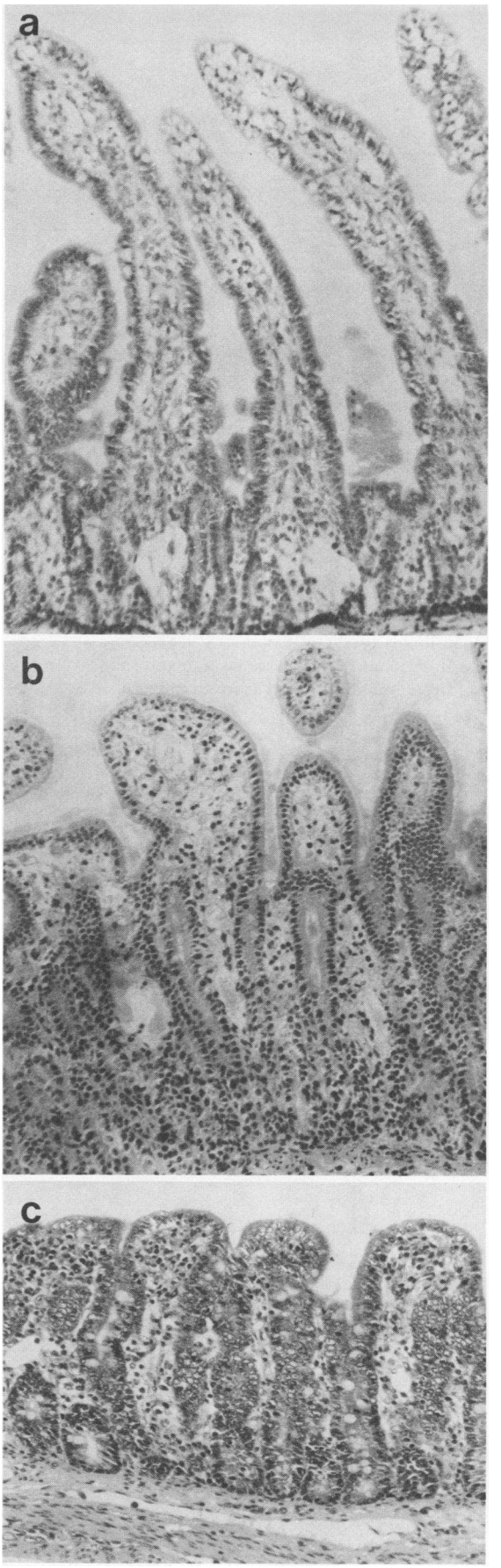

FIGURE 1 Light photomicrographs of upper jejunal mucoas (hematoxylin and eosin, magnification ×64, photographic reduction ×50%). (a) Control piglet showing normal villi; (b) HRV-infected piglet, moderate structural damage, blunted villi, increased crypt depth, cuboidal epithelial cells, and inflammatory cell infiltrate in the lamina propria. (c) HRV-infected piglet, severe structural damage, flattened villi, deep crypts, cuboidal epithelial cells, and inflammatory infiltrate in the lamina propria.

TABLE II

Ion Fluxes in Short-Circuited Piglet Jejunual Epithelium In Vitro*

		Na^+			Cl^-		
		M → S‡	S → M§	Net	M → S‡	S → M§	Net
	n						
Basal experimental conditions							
Control	9	10.83±0.73	12.62±0.78	−1.79±0.35	7.17±0.25	10.75±0.42	−3.58±0.38
Infected	11	6.59±0.34	8.68±0.29	−2.09±0.39	4.01±0.26	8.09±0.32	−4.08±0.40
P		<0.001	<0.001	NS	<0.001	<0.001	NS
Glucose added							
Control	9	14.56±0.71	12.46±0.52	2.10±0.50	9.25±0.40	12.19±0.60	−2.94±0.57
Infected	11	9.46±0.57	10.44±0.38	−0.98±0.46	5.26±0.31	9.17±0.33	−3.91±0.39
P		<0.001	<0.010	<0.001	<0.001	<0.001	NS
Theophylline added after glucose							
Control	9	18.12±1.02	17.76±1.11	0.36±0.77	14.61±1.21	19.95±1.58	−5.34±1.26
Infected	11	9.11±0.65	11.45±0.63	−2.34±0.40	7.53±0.55	14.48±0.82	−6.95±0.55
P		<0.001	<0.001	<0.010	<0.001	<0.025	NS

* Mean fluxes (μeq/cm² per hour)±1 SE.

‡ Unidirectional flux from mucosa to serosa.

§ Unidirectional flux from serosa to mucosa.

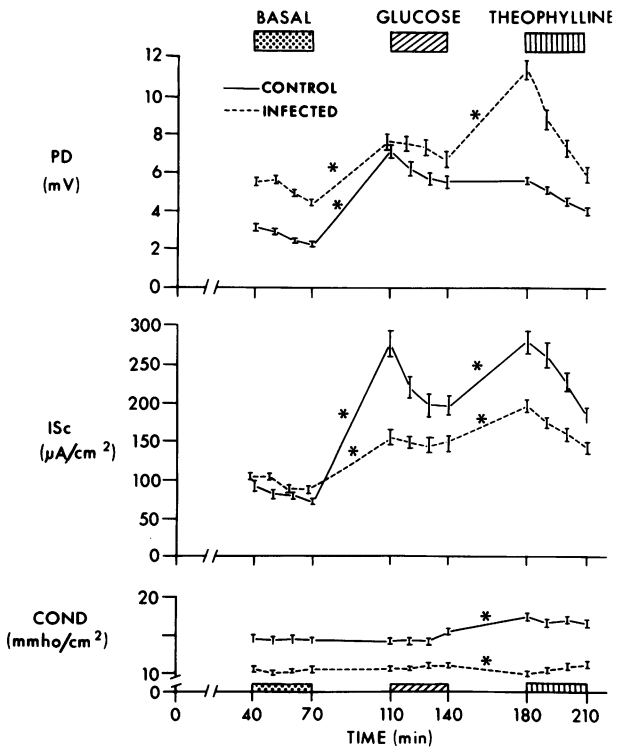

FIGURE 2 PD, ISc, and conductance (COND) in jejunal epithelium from HRV-infected and control pigs under basal conditions, with 30 mM glucose added, with 10 mM theophylline added after glucose. Values plotted as means ±1 SE. * $P < 0.001$.

rhea, particularly in the face of continuing carbohydrate consumption. These studies did not assess sugar absorption directly, but previous clinical studies have failed to detect a significant role for sugar malabsorption in the acute phase of HRV enteritis (29, 30).

Although cAMP is known to mediate active intestinal secretion in a number of diseases, including the enterotoxigenic diarrhea of cholera (31, 32), concentrations in the villus epithelium of HRV animals were normal. Our assays could have missed high concentrations of cAMP in crypt cells where Field has suggested neutral NaCl secretion is normally stimulated (33). However, in short-circuited chambers, epithelium from HRV-infected piglets did respond to theophylline, further supporting the absence of any preexisting cAMP stimulation. Unlike the pattern observed consistently in rabbit ileum (33, 34) and the jejunum of older pigs (15), the present control mucosa to serosa Na^+ flux failed to decrease in response to theophylline. This latter observation might be related to the very young age of the animals in the present study, but it is unexplained and warrants further study.

Our data suggest that defective epithelial function in this viral enteritis is due primarily to retarded differentiation of uninfected enterocytes migrating at an accelerated rate from the crypts after the virus has invaded villus cells. Enterocytes isolated from villi during acute diarrhea had an enzyme profile typical of normal crypt cells, rich in thymidine kinase

TABLE III

Disaccharidase and Na^+K^+ATPase Activities in Homogenates of Intestinal Mucosa*

		Duodenum		Upper jejunum		Ileum	
		Mean	Range‡	Mean	Range‡	Mean	Range‡
	n						
Lactase, *U/g protein*							
Control	9	43.0	29.4–62.9	144.0	122.6–169.1	37.2	27.8–49.7
Infected	11	9.7	8.3–11.3	11.8	10.1–13.7	3.8	3.3–4.4
P		<0.005		<0.001		<0.001	
Sucrase, *U/g protein*							
Control	9	4.6	4.2–4.9	23.8	17.6–32.7	16.5	14.0–19.4
Infected	11	0.2	0.1–0.2	2.1	1.8–2.6	10.3	7.8–13.7
P		<0.001		<0.001		NS	
Maltase, *U/g protein*							
Control	9	10.4	8.7–12.4	89.0	79.2–100.0	32.4	25.0–41.9
Infected	11	8.9	7.0–11.3	53.3	45.5–62.4	35.7	29.1–44.5
P		NS		<0.025		NS	
Na^+K^+ATPase, *U/mg protein*							
Control	9	2.0	1.8–2.3	2.6	2.3–3.0	2.1	1.9–2.4
Infected	11	1.0	0.8–1.4	0.8	0.6–1.1	2.2	2.0–2.4
P		<0.025		<0.005		NS	

* Disaccharidase activities are expressed as micromoles of substrate hydrolyzed/minute per gram protein, and Na^+K^+ATPase activity as micromoles of phosphate produced/hour per milligram protein.

‡ Data expressed as antilog of log of mean±1 SE.

but poor in sucrase (35). Recent studies in rats have shown that sugar-coupled Na^+ transport is not developed in normal crypt cells and that it develops as these cells differentiate and mature during their migration onto villi (36). In most pigs, viral antigen could not be found in the epithelium when diarrhea was severe, because by then, presumably, infected cells had been shed into the lumen. Probably the disease had evolved more slowly in these four animals in which virus antigen was detected; they had developed symptoms relatively late and had little or no diarrhea when they were killed. Clearly, the mechanisms of diarrhea in this invasive viral enteritis differ from the cAMP-mediated enterotoxigenic diarrheas caused by *Vibrio cholerae* (31, 32) and some strains of *Escherichia coli* (37). However, the present findings do resemble very closely our earlier observations made on piglets infected with transmissible gastroenteritis virus (15, 16). This invasive coronavirus enteritis occurs naturally in swine; when administered to young pigs, the virus causes an illness similar to, but more uniform and severe than, HRV enteritis of pigs (13, 14). The major transport defect identified, as in HRV enteritis, is the failure of sodium flux to respond to glucose (15, 16). When diarrhea is most severe, 40 h after infection, glucose-stimulated Na^+ transport in the upper intestinal epithelium is most defective (16), virus-infected enterocytes have been shed from the epithelium, and the enzyme pattern of

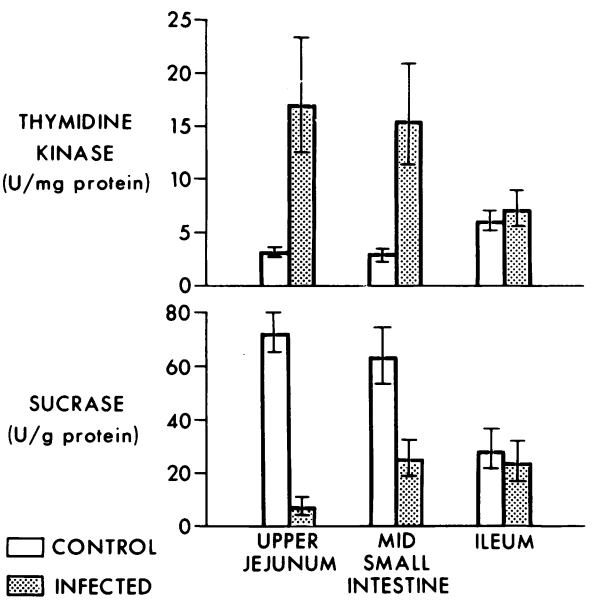

FIGURE 3 Thymidine kinase and sucrase activities in isolated villus enterocytes from three regions of the small intestine of control and HRV-infected pigs. Values are expressed as antilog of log of mean ±1 SE. Thymidine kinase activity is expressed as picomoles of thymidine phosphate reduced/minute per milligram protein, and sucrase activity as micromoles of substrate hydrolysed/minute per gram protein. Thymidine kinase increased; sucrase decreased ($P < 0.01$) in jejunum and mid-small intestine, but not ileum of HRV-infected pigs.

enterocytes lining the villi is characteristic of relatively undifferentiated crypt cells (16).

It should not be assumed that all invasive enteritides have a similar impact on intestinal function. However, a consistent pattern of intestinal damage has emerged from the study of two distinct enteric viral infections, one of them a major pathogen among infants and children. This information raises important questions concerning the active treatment of young patients with HRV enteritis. Because failure of enterocytes to differentiate fully as they migrate to intestinal villi appears to be a major determinant of disordered transport, factors such as malnutrition that may influence this maturation process should be carefully assessed and promptly attended to. Oral glucose electrolyte solutions designed in recent years for the active treatment of cholera (38) have demonstrated the value of early oral fluids in treating not only cholera patients but many with unspecified severe diarrhea (39). Because viral enteritis differs from cholera in many important respects, an oral glucose electrolyte mixture formulated specifically for HRV disease should be subjected to clinical trials.

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