Digestive Diseases and Sciences, Vol. 40, No. 10 (October 1995), pp. 2170–2176

Intestinal Morphology and Cytokinetics in Pancreatic Insufficiency

An Experimental Study in the Rat

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Intraluminal pancreatic enzymes influence intestinal function, adaptation, and susceptibility to injury. These effects may be mediated partly through changes in the rate of epithelial cell turnover. We assessed intestinal morphology and cytokinetics in a rat model of exocrine pancreatic insufficiency that does not alter anatomic relationships or animal growth. Pancreatic duct occlusion was performed by applying metal clips on both sides along the common bile duct. Control animals underwent sham-operation with exposure and manipulation of the pancreas without duct occlusion. Twelve days later, pulse labeling with tritiated thymidine was performed, and mitotic arrest was induced with colcemid. Groups of animals were sacrificed at 0 and 2 hr after colcemid injection. Specimens for histopathology, morphometry, and autoradiography were obtained from duodenum, proximal jejunum, distal jejunum, and ileum. Labeling index, grain counts, mitoses per crypt, cells per crypt, cells per villus, crypt depth, villus height, and number of goblet cells per villus were used as end points. Pancreatic duct occlusion resulted in increased labeling index across intestinal segments relative to sham-operated controls ($P < 0.01$) and increased labeling index and mitotic rate in distal compared to proximal intestine ($P < 0.05$). Grain-count histograms were similar in the two experimental groups. There were no significant morphologic differences between pancreatic duct-occluded animals and controls. Exocrine pancreatic insufficiency increases crypt cell proliferation in distal small intestine but does not alter the duration of S phase. These changes are most likely due to an increase in the size of the proliferative compartment and may be partly responsible for changes in small bowel function and response to injury.

KEY WORDS: pancreatic insufficiency; cytokinetics; small intestine; intestinal adaptation.

The amount and relative distribution of intraluminal pancreaticobiliary secretions (PBS) affect brush border membrane enzyme activity (1–4), mucosal morphology (5, 6), and adaptive responses to intestinal resection and transposition (7–9). Intraluminal factors also influence the incidence and severity of injury from endotoxinemia, ischemia, radiation, and hyperthermia (10–14). Whether these effects are due to a direct action of intraluminal contents on the mucosa, mediated through local and circulating gastrointestinal hormones, or secondary to changes in structure or epithelial cell turnover, is not known.

Several investigators have examined the combined and separate effects of pancreatic secretions, bile, and food on the intestinal mucosa. Most of these studies have been performed in animal models that involve

Manuscript received October 5, 1994; revised manuscript received March 24, 1995; accepted June 29, 1995.
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significant modification of intestinal anatomy (15, 16) or pharmacological and dietary manipulations (17, 18). Relative changes in segmental distribution of bile, pancreatic enzymes, and food, as well as the influence on nutritional status of the experimental animal, are confounding factors in such models.

We have developed and validated a rat model of exocrine pancreatic insufficiency that does not involve pancreaticobiliary diversion, biliointestinal anastomosis, or intestinal resection or transposition (19). This model provides the means to study the effects of pancreatic secretions specifically, while maintaining normal biliary secretion and intestinal continuity. Previous data from our laboratory suggest that changes in crypt cell proliferation may be involved in the mechanisms of some of the functional alterations and responses to injury observed in pancreas-insufficient animals. To test this hypothesis, the present study systematically examined the effect of exocrine pancreatic insufficiency on mucosal morphology and crypt cell kinetics throughout the small intestine.

MATERIALS AND METHODS

Animals. Fifty 180 to 200-g male Sprague-Dawley rats (Mollegaard Breeding, Lille Skensved, Denmark) were housed in groups of three to four with ambient air temperature 22 ± 1°C, relative humidity 55 ± 5%, and a 12/12-hr dark–light cycle with light from 06:30 to 18:30 hr. The animals had free access to standard rat chow (R3, Ewos, Sodertalje, Sweden) and tap water. The experiments were started after a one-week acclimatization period.

Measurements of Mitotic Rate with Stathmokinetic Method. An experiment with 20 rats was first performed to establish the optimal dose and time for assessment of metaphase accumulation rate in subsequent experiments.

The rats were injected with 0.1% colcemid (Demecolchin, Ciba-Geigy). The injection was given intraperitoneally at a dose of 6 mg/kg between 08:30 and 09:00 hr. Exactly 0, 1, 2, 3, and 4 hr after injection, groups of four rats were anesthetized with fluanison/fentanyl (Hypnorm, Janssen Pharmaceuticals, Beerse, Belgium) and midazolam (Dormicum, Roche, Basel, Switzerland), the entire small intestine was removed, and the animals were sacrificed by induction of bilateral open pneumothorax.

The duodenum from pylorus to ligament of Treitz was excised. The remaining small intestine was divided into three segments of equal length (proximal jejunum, distal jejunum, and ileum). Tissue samples, 2 cm in length, were taken from the middle of each segment, opened along the antimesenteric border, washed in saline, mounted on a piece of cucumber (to ensure proper orientation during processing and embedding), fixed in 4% buffered formalin, and embedded in paraffin. Slides were cut at 4 μm and stained with hematoxylin and eosin.

The number of metaphases was counted in longitudinally sectioned crypts (crypts in which the lumen, top, and base were seen). Twenty crypts per section were counted using a light microscope with a 40× objective. The arithmetic mean of the 20 crypts was considered a single value for statistical calculations.

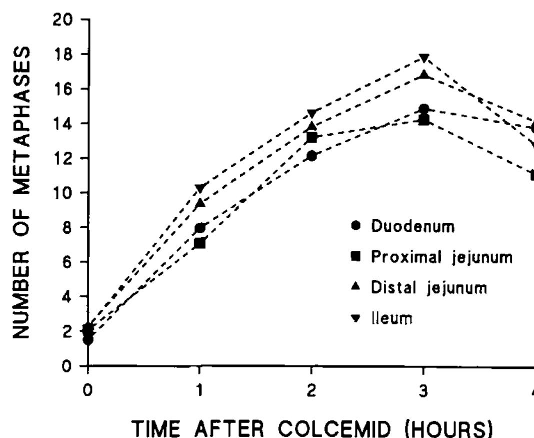

Fig 1. Average number of metaphases in the small intestine as a function of time after colcemid injection: near-linear increase is observed in all segments up to 3 hr after injection.

The metaphase accumulation experiment showed a linear increase in the number of metaphases up to 3 hr after injection in all intestinal segments, with a distinct drop between 3 and 4 hr (Figure 1). An accumulation period of 2 hr was therefore considered optimal for the colcemid dose and used for the subsequent experiments. The number of mitoses accumulated during this period, minus the mean number in the appropriate intestinal segment and group of control animals (pancreatic duct occluded or sham operated, sacrificed at 0 hr), was taken as a measure of the mitotic rate in the subsequent stathmokinetic experiments.

Pancreatic Duct Occlusion. Pancreatic duct occlusion was performed in 20 rats as reported previously (19). After overnight fasting, the rats were anesthetized with Hypnorm-Dormicum. An upper midline abdominal incision was made, the duodenum was mobilized, and small metal hemostatic clips were applied along both sides of the entire length of the common bile duct from the duodenum to the confluence of the hepatic ducts (Figure 2). This procedure occludes the multiple pancreatic ducts unique to the rat without the need for a biliointestinal drainage procedure. After an initial drop in body weight secondary to surgery

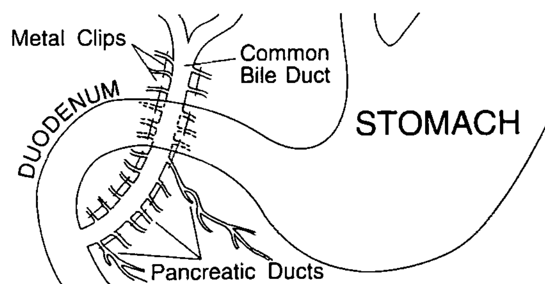

Fig 2. Pancreatic duct-occlusion model to induce exocrine pancreatic insufficiency without altering biliary or intestinal anatomy. The numerous pancreatic ducts are occluded by application of metal clips along both sides of the common bile duct.

and induced pancreatitis, the animals exhibit normal growth. The operation causes a >95% reduction in maximum cholecystokinin-stimulated trypsin secretion, bile flow is undisturbed, and blood glucose levels remain within normal limits (19).

Ten animals underwent a sham operation consisting of mobilization of the duodenum and exposure and manipulation of the pancreas, but without occlusion of the pancreatic ducts.

Tissue Procurement and End Points. At 08:00 hr, 12 days after pancreatic duct occlusion or sham operation, the animals received an intraperitoneal injection of colcemid (6 mg/kg) and undiluted tritium-labeled thymidine ([^{3}H]TdR) (Amersham, Little Chalfont, England) with specific activity 5.0 Ci/mmol (1 μCi/g body weight, total injection volume approximately 0.3 ml). Exactly 2 hr after injection, intestinal samples were procured and processed as described earlier.

The following end points were assessed: villus height, number of cells per villus, number of goblet cells per villus, crypt depth, and number of cells per crypt. Epithelial cytokinetics were examined by autoradiography to determine labeling index (relative number of cells in S phase) and grain counts (as an estimate of the duration of S phase) as well as by the metaphase arrest technique to measure the mitotic rate.

Morphometry. Crypt depth and villus height were measured with a Kontron image analysis system with Video Plan software (Kontron Electronics, Munich, Germany). Crypts were assessed only if cut vertically through their longest axis, ie, if the top, Paneth cells at the base, and entire lumen were visible. Villi were measured only if erect and located between two complete crypts. In each animal, 20 complete crypts and villi were measured. The arithmetic mean for each animal was considered as one value and used for further calculations.

Number of epithelial cells per villus and crypt and goblet cells per villus were counted by light microscopy. The "average cell size index" was calculated by dividing the number of cells by the villus height or crypt depth, as appropriate.

Autoradiography and Metaphase Counting Technique. Unstained sections on slides were dipped in Kodak NTB-2 liquid film emulsion diluted 1:1 with distilled water, exposed in total darkness at 4°C for 14 days, developed, and counterstained with hematoxylin. Number of cells per crypt (excluding Paneth cells), number of metaphases per crypt, and number of labeled cells per crypt were determined in 20 crypts per specimen. The distribution of number of grains per labeled cell (grain counts) were obtained from 10 crypts per specimen from distal jejunum and ileum (ie, the segments with significant differences in mitotic count and/or labeling index between pancreatic duct-occluded animals and controls). In each crypt, the mean number of grains in 20 labeled cells was determined.

Statistical Calculations. Statistical calculations were performed with BMDP PC90 (BMDP Statistical Software, Inc., Los Angeles, California) and Statistix Version 3.5 (Analytical Software, St. Paul, Minnesota). Two-tailed significance levels were used throughout, and no pooling of data was performed. Analysis of variance (ANOVA) with repeated measures was performed on morphometric and cytokinetic data. Treatment was used as grouping factor (PDO or sham-operation) and intestinal segment as within factor (duodenum, proximal jejunum, etc.). Tukey's HSD was used for *post hoc* multiple comparison when significant F values were obtained with the ANOVA. Student's t test was used when appropriate for assessment of the difference between two group means. The difference in distribution of grain counts was assessed using contingency table analysis.

RESULTS

Pancreatic duct occlusion did not change mucosal morphology, but significantly altered small bowel crypt cell kinetics. The morphologic end points are shown in Figure 3. Both sham-operated controls and pancreatic duct-occluded animals exhibited normal proximal to distal gradient, that is, villus height and number of epithelial cells per villus decreased and average number of goblet cells per villus increased from duodenum to ileum. There was no significant proximal to distal gradient for crypt depth measurements or mean number of epithelial cells per crypt. There was no significant difference between pancreatic duct-occluded animals and sham-operated controls with regard to the number of epithelial cells per crypt, number of epithelial cells per villus, mean crypt depth, or mean villus height. Pancreatic duct-occluded animals also did not differ significantly from controls with regard to absolute or relative number of goblet cells per villus or cell size index (data not shown).

Intestinal crypt cell kinetics data in pancreatic duct-occluded animals and sham-operated controls are shown in Figure 4. Repeated measures ANOVA using labeling index as the dependent variable revealed that pancreatic duct-occluded animals had significantly higher average labeling index across intestinal segments than sham-operated controls (P = 0.01). The difference in crypt labeling index among intestinal segments was also statistically significant (P < 0.01), whereas the interaction between intestinal segment and experimental group was not (P = 0.8). Pancreatic duct occlusion and intestinal segment did not emerge as significant factors when mitotic rate was used as dependent variable (P = 0.4 and P = 0.06, respectively).

Sham-operated control animals showed no significant difference or trend among intestinal segments (proximal to distal) with regard to mitotic rate or labeling index. In contrast, ANOVA with *post hoc* Tukey's HSD test revealed that pancreatic duct-occluded animals had increased mitotic rate (P < 0.05) and labeling index (P < 0.05) in ileum compared to proximal intestine. Pairwise group compar-

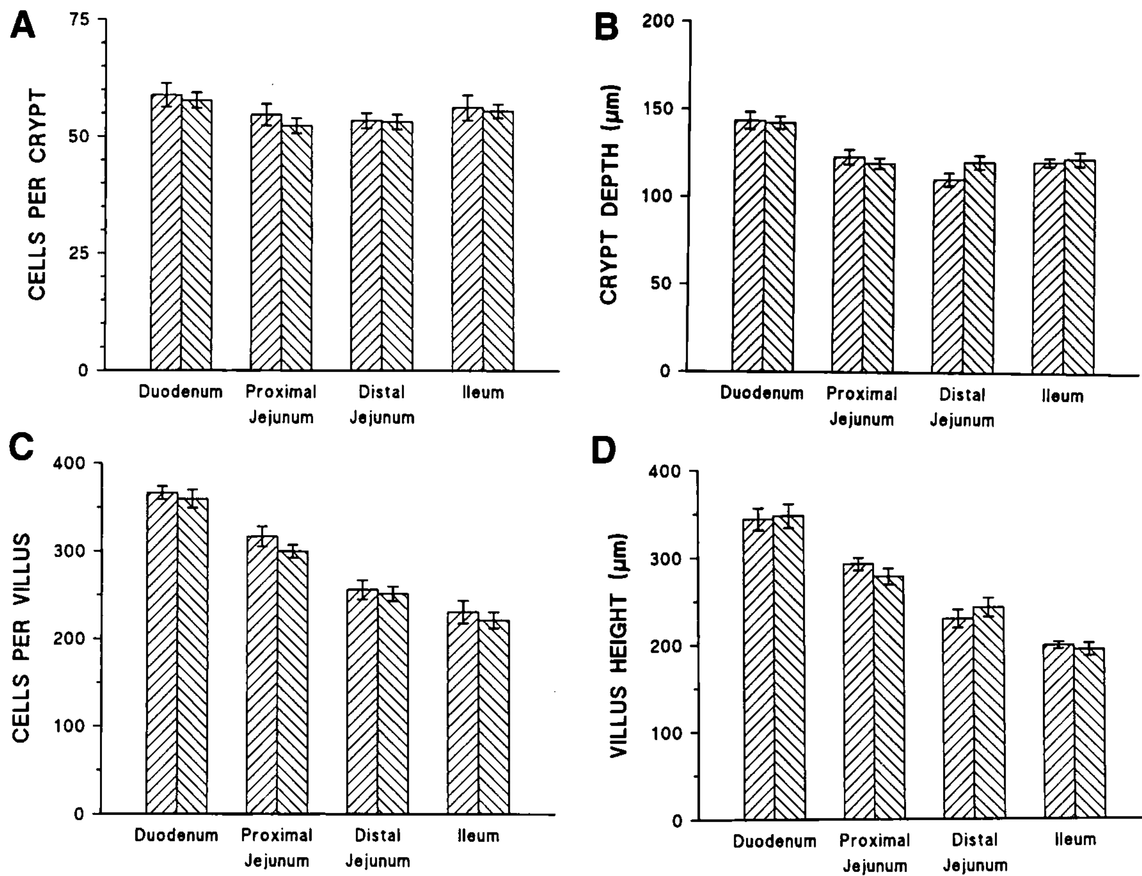

Fig 3. Morphological data and cell counts in the small intestine of pancreatic duct-occluded animals (right bars) and sham-operated controls (left bars). Error bars: ± SEM. (A) Average number of epithelial cells per crypt; (B) average crypt depth; (C) average number of epithelial cells per villus; (D) average villus height.

ison of intestinal segments showed increased labeling index in distal jejunum ($P < 0.01$) and ileum ($P < 0.05$) of pancreatic duct-occluded animals relative to controls, whereas the difference in average number of mitoses per crypt was just below the level of statistical significance ($P < 0.06$).

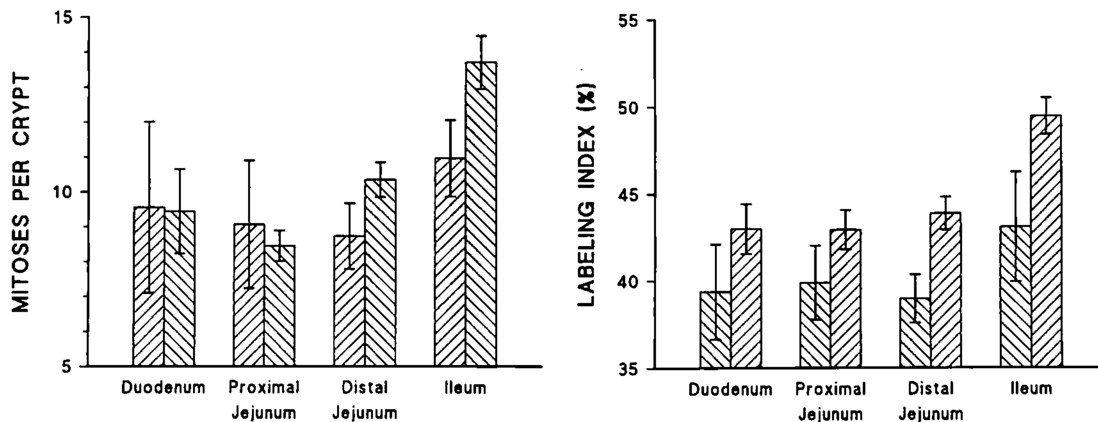

Fig 4. Labeling index (left panel) and increase in the number of mitoses per crypt from 0 to 2 hr after colcemid injection (right panel) in the small intestine of pancreatic duct-occluded animals (right bars) and sham-operated controls (left bars). Error bars: ± SEM.

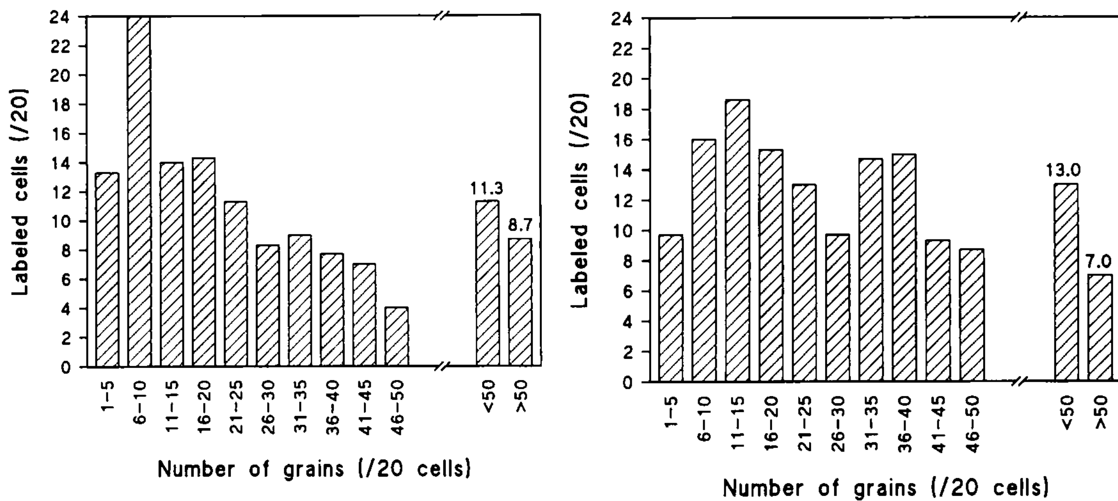

Fig 5. Grain count data from ileum of sham-operated controls (left panel) and pancreatic duct-occluded rats (right panel).

There was no significant difference in the distribution of grains in labeled cells between the two treatment groups. The results of grain counts in ileum are depicted in Figure 5.

DISCUSSION

In addition to their role in digestion of nutrients, pancreatic secretions influence intestinal structure and function, as well as expression of injury caused by ischemia, hyperthermia, and radiation (11–14, 20). Some of these properties may be explained in terms of changes in epithelial cell kinetics that affect development of and/or recovery from mucosal damage.

The influence of intraluminal factors on the intestine has been examined in surgical animal models that deprive specific segments of pancreatic secretions, bile, or food (8, 15, 16, 21, 22), or transpose distal intestine to a more proximal position. Other models use dietary and/or pharmacologic manipulation to induce pancreatic insufficiency (23, 24). Diversion of pancreaticobiliary secretions (PBS) or food away from the normal route, resection, or transposition of intestinal segments create conditions that differ from those encountered in isolated exocrine pancreatic insufficiency. This may explain some of the apparent inconsistencies in the literature.

Pancreatic secretions induce trophic and functional changes if shunted to the distal bowel, whether the distal segment is in continuity (ie, receives food) or not (2, 5, 8, 22). In contrast, a lack of pancreatic enzymes throughout the bowel is associated mainly with functional alterations, and it is less clear to what extent mucosal morphology and cell kinetics are altered. Pancreatic insufficiency causes increased brush border membrane enzyme activity in distal small bowel (18, 25). This is probably a result of altered enzyme degradation rates but may also be a result of changes in epithelial cell maturation or turnover (26). Although there is some increase in mucosal wet weight in pancreatic insufficiency (18), structural changes are less evident by morphometry (27). Thus far, no systematic cytokinetic study has been performed using models of isolated pancreatic insufficiency.

Our surgical model does not require dietary or pharmacologic manipulation, intestinal transposition, or diversion of PBS. The animals are in an essentially normal physiologic state and exhibit normal growth after an initial lag-phase caused by postoperative pancreatitis. The procedure causes total histopathologic atrophy of the exocrine pancreas without affecting Langerhans' islet morphology. Stimulated trypsin output is reduced by >95%, there is no biochemical evidence of biliary obstruction, and blood glucose levels remain within the normal range (19).

The present study used well-established morphometric and cytokinetic methods to assess mucosal structure and cell turnover: pulse labeling with tritiated thymidine was used to estimate the relative size of the proliferative pool, cell division rate was assessed with a metaphase arrest technique, and relative changes in S-phase duration were estimated by grain counts.

Colcemid-induced accumulation of metaphases is optimal for measuring the mitotic rates during a given time period, provided that cells enter mitosis unper-

turbed, followed by a complete block in metaphase (28). An excessive drug dose reduces the entrance into mitosis, whereas an insufficient dose causes cells to "leak" out of mitosis. The dose used in the present experiment was chosen based on previous experience with this technique in our laboratory. Since colcemid blocks mitosis during the 2-hr interval between injection and sacrifice, the recorded number of cells having incorporated tritiated thymidine will reflect the number of cells in S phase at the time of injection 2 hr previously.

In the present study, pancreatic insufficiency caused increased cell turnover in the distal small intestine. Experimental animals exhibited a significant proximal-to-distal gradient in labeling index and mitotic rate that was not seen in controls. There was also a significant increase in labeling index in the distal intestine of pancreatic duct-occluded animals compared to controls. These findings are in accordance with data in the literature indicating that changes induced by pancreatic insufficiency are greater in distal than in proximal small bowel (18). A possible explanation of these observations is that the concentration of proteolytic enzymes is greater in distal than in proximal small bowel in the normal rat (29). Thus, absence of pancreatic secretions has greater impact in distal jejunum and ileum than in duodenum and proximal jejunum. The situation may be reversed in humans, where the concentration of pancreatic enzymes decreases toward the ileum (30). It is also possible that the absence of pancreatic secretions requires greater adaptation in the distal than in proximal segments due to compensatory production of proteases of mucosal origin in the jejunum.

The sham operation used in the present study is clearly less traumatic than the pancreatic duct-occlusion operation. It is thus conceivable that some observed differences may be attributed to pancreatitis rather than to pancreatic insufficiency. However, in spite of significant pancreatitis in the early postoperative period, absolute body weight, rate of weight gain, and intestinal mucosal wet weight at days 10–15 are not significantly diminished compared to control values (19, 25). The absence of morphological differences in the mucosa in the present study also indicates that, most likely, the observed cytokinetic changes are a result of pancreatic insufficiency.

Increased crypt cell proliferation rate may occur as a result of decreased turnover time and/or increased size of the proliferative cell pool. No significant difference in grain counts was observed, ie, the increased labeling index was not a result of increased S-phase duration. Our findings may thus be explained in terms of shortening of the cell cycle time and/or an increase in the relative size of the proliferative pool. However, because cell cycle time in the small intestine is very short, expansion of the proliferative pool is more likely.

Our findings may apply to possible mechanisms of enteroprotection by removal or neutralization of intraluminal pancreatic secretions. Based on the present study, we surmise that such protection is conferred by a combination of eliminating the detrimental effect of pancreatic proteases on the injured bowel mucosa and by accelerating regeneration of the epithelium.

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