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Oral Insulin Enhances Intestinal Regrowth Following Massive Small Bowel Resection in Rat

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Experimental studies have suggested that insulin (INS) plays an important role in small intestinal growth and development. In the present study we investigated the effect of oral INS on structural intestinal adaptation and enterocyte proliferation and loss via apoptosis in a rat model of short bowel syndrome (SBS). Male Sprague–Dawley rats were divided into three experimental groups: sham rats underwent bowel transection, SBS rats underwent 75% small bowel resection, and SBS-INS rats underwent bowel resection and were treated with oral INS given in the drinking water from the 3rd to the 15th postoperative day. Parameters of intestinal adaptation (bowel and mucosal weight, mucosal DNA and protein, villous height, and crypt depth), enterocyte proliferation, and apoptosis were determined on day 15. SBS-INS rats demonstrated a significant increase (vs SBS rats) in jejunal and ileal overall bowel and mucosal weight, ileal mucosal DNA and protein, ileal villous height, and crypt depth. SBS-INS rats also showed an increased cell proliferation index in jejunum and ileum and decreased apoptotic index in jejunum compared to SBS animals. In conclusion, in a rat model of SBS, oral INS strongly enhances intestinal adaptation. Possible mechanisms may include increased cell proliferation and decreased enterocyte loss via apoptosis.

KEY WORDS: short bowel syndrome; intestinal adaptation; oral insulin.

Despite the availability of total parenteral nutrition, high-quality venous access devices, improved technique in surgery, progress in neonatal care, and advances in anesthesia and medical treatment, the morbidity and mortality in patients suffering from short bowel syndrome (SBS) remains strikingly high (1). Evidence suggests that the survival rate after massive small bowel resection depends on the ability of the residual bowel to adapt. "Adaptation" is the term applied to progressive recovery from intestinal failure following a loss of intestinal length (2, 3). The remaining intestine dilates, elongates, and thickens, and villous height and crypt depth increase, leading to mucosal hyperplasia and increased absorptive surface area (structural adaptation). In addition, increased nutrient absorption by isolated enterocytes occurs (functional absorption). Over the past decades, much research has focused on factors that may promote intestinal regrowth. There is a growing body of evidence suggesting that different hormones and peptide growth factors may stimulate postresection adaptive hyperplasia (2, 3).

The peptide growth factors involved in intestinal homeostasis are classified into several discrete families on the basis of structural homologies and disparities, which include the epidermal growth factor family, transforming growth factor-β family, insulin (INS)-like growth factor (IGF) family, and fibroblast growth factor family. In addition, a smaller number of peptide growth factors without structural similarities to the previously described families have also been identified and include hepatocyte growth factor and platelet-derived growth factor (4).

The INS-like growth factor family includes three peptides: INS, INS-like growth factor I (IGF-I), and INS-like growth factor II (IGF-II). Several experimental studies

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have suggested that both IGF-I and IGF-II are involved in the modulation of growth and differentiation of normal small bowel (5, 6) as well as in the bowel following massive resection (7). Lemmey and coworkers have demonstrated a positive effect of IGF-1 on body weight gain (8) and intestinal absorptive function (9) following bowel resection in a rat model. Ziegler and colleagues have demonstrated that the ileal IGF-I mRNA expression in rats rose nearly twofold during intestinal adaptation after bowel resection, which was augmented with IGF-I administration (10). Lukish and associates (11) have demonstrated that EGF and IGF-1 increase substrate absorption after small bowel resection in rats, and this increase in absorption persisted after discontinuation of these growth factors.

Although a positive effect of IGF (I and II) on intestinal adaptation has been well described previously, little evidence exists that INS may affect intestinal growth following bowel resection. Oral INS administration is an unusual route for this hormone. However, the fact that INS is present in human and pig colostrum and mature milk and that INS receptors are present on both luminal and basolateral membranes of the enterocyte may suggest its potential role in small intestinal growth and development. Recent experimental studies have demonstrated that INS has enterotrophic effects on the normal gut (12–14). The purpose of this study was to evaluate the effects of oral INS on structural intestinal adaptation following massive small bowel resection in rat and to determine the mechanisms by which INS influences mucosal hyperplasia as well as its effect on enterocyte proliferation and apoptosis.

MATERIALS AND METHODS

Animals. The Rappaport Faculty of Medicine (Technion, Haifa, Israel) Institutional Animal Care and Use Committee approved the animal facilities and protocols. Briefly, male rats weighing 240–260 g were kept in individual stainless-steel cages at a constant temperature and humidity, and a 12-hr light–dark cycle was maintained. Rats were fasted for 12 hr before the experiment with free access to water. Animals underwent one of two surgical procedures: bowel transection and reanastomosis or 75% bowel resection and anastomosis. General anesthesia was induced with pentobarbital (40 mg/kg IP). Using sterile techniques, the abdomen was opened using a midline incision. In sham rats, simple bowel transection and end-to-end reanastomosis were performed 15 cm proximal to the ileocecal junction. In SBS animals, the small bowel was resected from a point 5 cm distal to the ligament of Treitz to a point 10 cm proximal to the ileocecal junction. Bowel continuity was restored by end-to-end anastomosis using 5-0 Vicryl interrupted sutures. For all operations, the abdominal cavity was closed in two layers with a running suture of 3-0 Dexon. Before closure of the abdomen, the rats were resuscitated with a 3-ml IP injection of warm normal saline. Rats were fasted for 24 hr but were allowed free access to water.

Experimental Design. Forty rats were randomly assigned to one of three groups: Group A rats underwent bowel transection (sham; $n = 14$) and were then fed regular chow diet; Group B animals underwent bowel resection (SBS; $n = 13$) and were then fed regular chow diet; and Group C rats (SBS-INS; $n = 13$) underwent bowel resection and were fed regular chow diet and water containing INS at concentration of 1 U/ml as described previously (14). Animals were followed for 14 days with monitoring of food and fluid intake and weight measurement. After 14 days, the animals were sacrificed after IP injection of pentobarbital (75 mg/kg).

Intestinal Adaptation Parameters. The small bowel was rapidly removed, rinsed with cold isotonic saline, and divided into two segments: jejunum proximal to anastomosis and terminal ileum. Each segment was weighed and cut longitudinally, bowel circumference was measured at three equidistant points as described by Dowling (15), and mean circumference was calculated. Mucosa was scraped using a glass slide, collected, and weighed. Bowel and mucosal weight was calculated per centimeter of bowel length per 100 g of body weight as described previously (16, 17). Although bowel length may change due to spasm or bowel distension, calculation per unit of bowel length is considered to be the gold standard for describing structural changes in the intestine. DNA and protein were extracted using TRIzol reagent as described by Chomczynski (18). DNA concentration was recorded spectrophotometrically and calculated per centimeter of bowel length. Final protein concentration was measured spectrophotometrically using a commercially available kit (Bio-Rad Protein Assay) and was calculated per centimeter of bowel length.

Histologic sections were prepared from the proximal jejunum and distal ileum and from comparable sites in control animals. Segments of small bowel were fixed for 24 hr in 10% formalin and processed into standard paraffin blocks. Five-micron tissue slices were stained with hematoxylin–eosin. These sections were studied microscopically using a micrometer eyepiece. Histologic images were loaded on a 760 × 570-pixel resolution buffer using a computerized image analysis system composed of a trichip RGB videocamera (Sony, Japan), installed on a light microscope (Zeiss, Germany) and attached to an IBM-compatible personal computer (Pentium III, MMX; 450 mHz, 125-MB RAM), equipped with a frame grabber. Images were captured, digitized, and displayed on a high-resolution, 17-in. color monitor. The villous height and crypt depth were measured using the Image Pro Plus 4 image analysis software (Media Cybernetics, Baltimore, MD, USA). Ten villi and crypts in each section were measured and the mean reading was recorded in microns.

Crypt Cell Proliferation and Enterocyte Apoptosis. Rats were injected with standard 5-bromodeoxyuridine (5-BrdU) labeling reagent (Zymed Laboratories, Inc., USA) at 1 ml per 100 g body weight 2 hr before sacrifice. Tissue slices (5 μm) were deparaffinized with xylene, rehydrated with graded alcohol, and stained with a biotinylated monoclonal anti-BrdU antibody system using a BrdU Staining Kit (Zymed Laboratories, Inc.). An index of proliferation was determined as the ratio of crypt cells staining positively for BrdU per 10 crypts.

Apoptosis of enterocytes was assessed by terminal deoxyuridine nick-end labeling (TUNEL) immunohistochemical assay using the I.S. Cell Death Detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). Five-micron-thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and microwave-pretreated in 10 mM citrate

buffer (pH 6.0) for 10 min. After washing in phosphate-buffered saline (PBS), the specimens were incubated in buffer containing a nucleotide mixture with fluorescein-labeled deoxy-UTP and TdT at 37°C for 1 hr. After washing, the slides were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min and were stained with antifluorescein antibody, Fab fragment from sheep, conjugated with horseradish peroxidase (converter-POD) at 37°C for 30 min. AES substrate (Zymed Laboratories, Inc.) was applied for color development. For each group, the number of stained cells was counted in 10 villi in the areas without necrosis. The apoptotic index was defined as the number of apoptotic TUNEL-positive cells per 10 villi. All measurements were performed by a qualified pathologist blinded as to the source of intestinal tissue.

Statistical Analysis. The data are expressed as the mean ± SE. The nonparametric Kruskal–Wallis ANOVA was used as indicted. $P < 0.05$ was considered statistically significant.

RESULTS

Body Weight. The sham-operated control rats (Group A) maintained a constant body weight during the first 4 postoperative days, then gradually increased in weight throughout the 2-week observation period (Figure 1). Resected rats (Groups B and C) demonstrated a significantly lower body weight from day 4 through day 14 after the operation compared to their sham-operated counterparts. SBS-INS rats (Group C) gained weight at a faster rate from day 7 through day 14 compared to SBS-untreated animals (Group B) ($P < 0.05$).

Macroscopic Bowel Appearance. Two weeks after bowel resection, there was an increase in intestinal thickness and diameter. Compared to sham animals (Group A), SBS rats (Group B) showed a significantly greater bowel circumference in jejunum and ileum (Table 1). Exposure to oral INS resulted in additional bowel enlargement. SBS-INS rats (Group C) demonstrated an increase in jejunal and ileal bowel circumference compared to SBS-untreated animals (Group B).

Overall mean bowel weight rose significantly in jejunum (fourfold increase; $P < 0.05$) and in ileum (twofold increase; $P < 0.05$) in SBS-rats (Group B) compared to sham animals (Group A) (Table 1). Following oral INS administration (Group C), SBS rats demonstrated an additional significant increase in jejunal (18%; $P < 0.05$) and ileal (40%; $P < 0.05$) overall weight compared to SBS-untreated animals.

Changes in mucosal weights were similar to those in bowel weights (Table 1). SBS rats (Group B) demonstrated a threefold increase in jejunal mucosal weight ($P < 0.05$) and twofold increase in ileal mucosal weight ($P < 0.05$) compared to sham animals (Group A). The oral INS-supplemented group (Group C) demonstrated an additional 33% increase in jejunal mucosal weight ($P < 0.05$) and almost twofold increase in ileal mucosal weight ($P < 0.05$) compared to SBS-untreated counterparts.

Mucosal DNA and Protein. Adaptation in residual bowel in the resected group (Group B) was manifested by a 2.7-fold increase in jejunal ($P < 0.05$) and a 1.6-fold increase in ileal ($P < 0.05$) DNA content compared to sham animals (Table 2). Oral INS supplementation resulted in

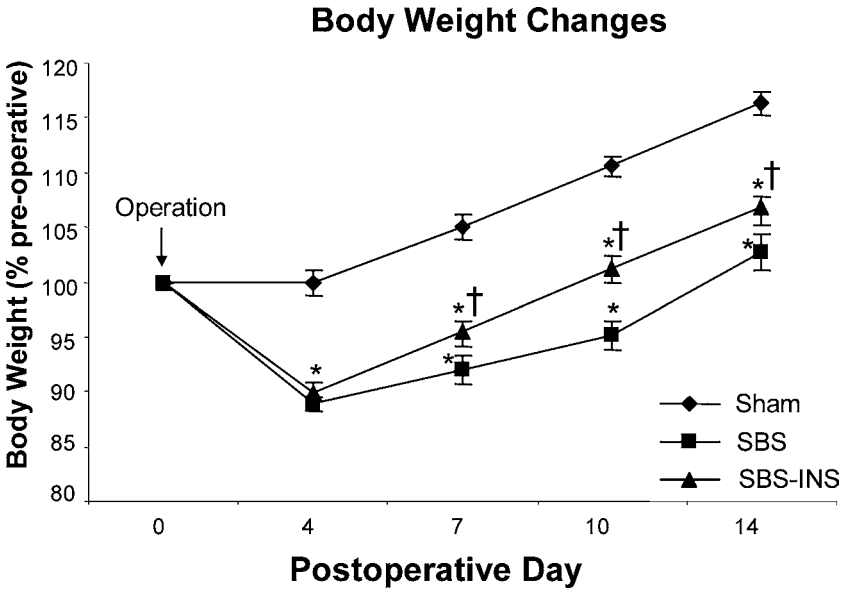

Fig 1. Body weight changes expressed as percentage of preoperative weight (mean ± SE) in control (sham) and resected rats untreated (SBS [short bowel syndrome]) or treated with oral insulin (INS). *$P < 0.05$, SBS vs sham rats; †$P < 0.05$, SBS-INS vs SBS rats.

TABLE 1. EFFECT OF BOWEL RESECTION AND ORAL INSULIN ON MACROSCOPIC BOWEL APPEARANCE IN RAT

Parameter	*Sham (Group A;* n = *14)*	*SBS (Group B;* n = *13)*	*SBS-INS (Group C;* n = *13)*
Bowel circumference (mm)			
Jejunum	7.9 ± 0.3	10.7 ± 1.3*	11.5 ± 0.2*†
Ileum	8.3 ± 0.2	9.7 ± 0.3*	10.5 ± 0.2*†
Bowel weight (mg/cm/100 g)			
Jejunum	19 ± 1	71 ± 5*	84 ± 5*†
Ileum	21 ± 1	43 ± 2*	60 ± 3*†
Mucosal weight (mg/cm/100 g)			
Jejunum	7.3 ± 0.3	24.4 ± 1.6*	32.3 ± 1.7*†
Ileum	6.8 ± 0.5	13.5 ± 1*	21.9 ± 1*†

Note. SBS, short bowel syndrome; INS, insulin.
*$P < 0.05$, SBS and SBS-INS vs sham.
†$P < 0.05$, SBS-INS vs SBS.

an almost twofold increase in ileal DNA content compared to SBS-untreated animals ($P < 0.05$).

Mucosal protein content increased significantly after bowel resection in both jejunum (threefold increase; $P < 0.05$) and ileum (1.4-fold increase; $P < 0.05$) (Table 2). Oral INS administration (Group C) induced an additional twofold increase in ileal ($P < 0.05$) mucosal protein content compared to SBS-untreated animals (Group B).

Microscopic Bowel Appearance. SBS rats (Group B) showed a marked increase in villous height in jejunum (785 ± 34 vs 529 ± 34 μm; $P < 0.05$) and ileum (672 ± 24 vs 462 ± 32 μm; $P < 0.05$) and in crypt depth in jejunum (209 ± 15 vs 163 ± 9 μm; $P < 0.05$) and ileum (172 ± 11 vs 147 ± 7 μm; $P < 0.05$) compared to sham animals (Group A) (Table 2). SBS-INS rats (Group C) demonstrated a 15% increase in ileal villus height ($p < 0.05$), a 15% increase in jejunal ($p < 0.05$) and 40% increase in ileal ($P < 0.05$) crypt depth compared to SBS-untreated animals (Group B).

Enterocyte Proliferation and Apoptosis. Bowel resection (Group B) resulted in a significant increase in enterocyte proliferation index in jejunum (258 ± 17 vs 154 ± 7 BrdU-positive cells/10 crypts; $P < 0.05$) and ileum (263 ± 15 vs 182 ± 9 BrdU-positive cells/10 crypts; $P < 0.05$) compared to sham animals (Figures 2 and 3). Oral INS administration (Group C) induced an additional 36% increase in proliferation index in jejunum ($P < 0.05$) and a 52% increase in proliferation index in ileum ($P < 0.05$) compared to SBS-untreated animals (Group B).

Significantly greater numbers of apoptotic cells appeared in the villi of jejunum (29 ± 7 vs 13 ± 4 TUNEL-positive cells/10 villi; $P < 0.05$) and ileum (33 ± 8 vs 14 ± 5 TUNEL-positive cells/10 villi; $P < 0.05$) in SBS rats (Group B) compared to sham animals (Figure 3).

TABLE 2. EFFECT OF BOWEL RESECTION AND ORAL INSULIN ON MUCOSAL DNA AND PROTEIN CONTENT AND MICROSCOPIC BOWEL APPEARANCE IN RAT

Parameter	*Sham (Group A;* n = *14)*	*SBS (Group B;* n = *13)*	*SBS-INS (Group C;* n = *13)*
Mucosal DNA (μg/cm/100g)			
Jejunum	13 ± 2	36 ± 5*	43 ± 4*
Ileum	11 ± 1	18 ± 2*	32 ± 5*†
Mucosal protein (μg/cm/100g)			
Jejunum	15 ± 1	51 ± 5*	56 ± 6*
Ileum	17 ± 2	24 ± 2*	49 ± 4*†
Villus height (μm)			
Jejunum	529 ± 31	785 ± 34*	758 ± 32*
Ileum	462 ± 32	672 ± 24*	776 ± 44*†
Crypt depth (μm)			
Jejunum	164 ± 9	209 ± 15*	236 ± 13*
Ileum	147 ±7	172 ± 11*	241 ±8*†

Note. SBS, short bowel syndrome; INS, insulin.
*$P < 0.05$ SBS and SBS-INS vs sham.
†$P < 0.05$ SBS-INS vs SBS.

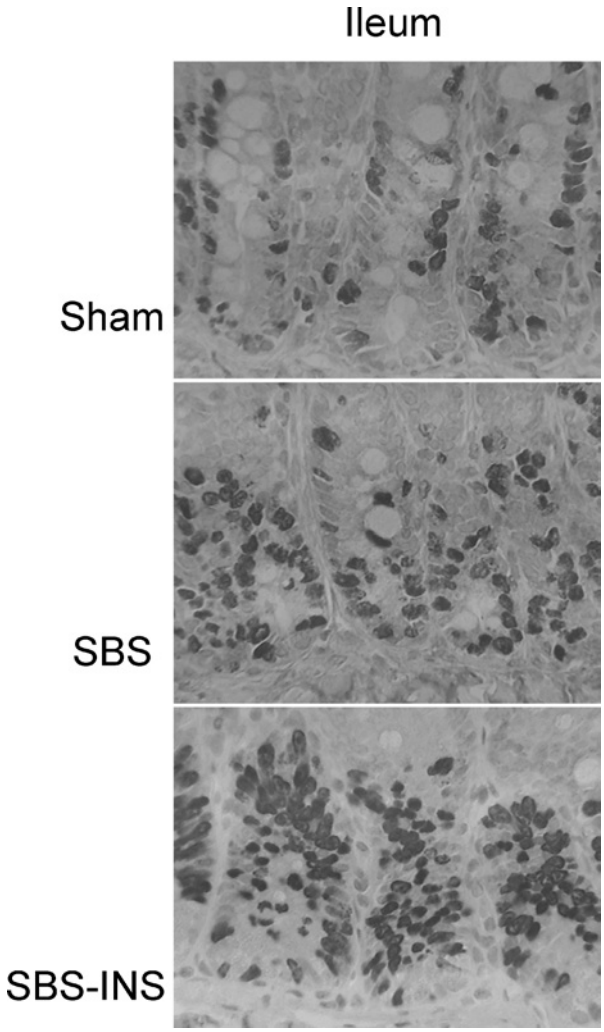

Fig 2. Effect of bowel resection and oral insulin (INS) on enterocyte proliferation. These representative sections demonstrate that cell proliferation is increased in rats following bowel resection (SBB) compared to sham animals (sham). Following administration of oral INS, short bowel syndrome (SBS) rats (SBS-INS) demonstrated a marked increase in number of proliferating cells compared to SBS-untreated animals.

Exposure to oral INS led to a significant decrease in apoptotic index in jejunum (11 ± 3 vs 29 ± 7 TUNEL-positive cells/10 villi; $P < 0.05$) and no change in apoptotic cell number in ileum compared to SBS-untreated animals (Group B).

DISCUSSION

This is the first study investigating the effects of oral INS as a gut-trophic agent following bowel resection in an animal model of SBS. Our results show that massive intestinal resection results in significant structural adaptation. This conclusion is supported by the observed increase in bowel and mucosal weight of the remnant bowel, increase in mucosal DNA and protein, and increase in villous height and crypt depth in this model. The decrease in body weight in resected rats may suggest a state of malnutrition despite the adaptation process. The changes in body weight and remaining small bowel morphology in the current report were similar to those observed in our previous experiments (16, 17).

Oral INS supplementation dramatically enhanced structural intestinal adaptation. This effect was much more significant than that observed previously following administration of other factors such as transforming growth factor-α (16) or a high-fat diet (17). Dietary INS supplementation significantly increased overall bowel and mucosal weight, which occurred with a synergistic increase in bowel circumference. However, an aproximately 10% increase in bowel diameter was accompanied by a 20–50% increase in overall bowel weight and 30–200% increase in mucosal weight in remaining segments. These data support the concept that mucosal hyperplasia rather than bowel enlargement is responsible for increased bowel and mucosal weights.

Oral INS significantly increased ileal mucosal DNA and protein. Parallel increases in mucosal DNA and protein indicate that the greater ileal mass of animals treated with oral INS can be attributed to cellular hyperplasia. Because the DNA and protein content is directly proportional to the mucosal cell number, these measurements exclude such factors as edema and vascular engorgement as being responsible for differences in mucosal mass. Increased mucosal proliferation in functioning intestine, as demonstrated by the increased cell proliferation index following oral INS administration, suggests an activated enterocyte turnover and may be considered as a main mechanism of mucosal hyperplasia in residual bowel. Increased villous height and crypt depth are the result of increased proliferation and accelerated migration along the villus and are a marker for an increased absorptive surface area. Most significant differences were observed in the terminal ileum, while hyperplasia in the proximal jejunum was less prominent. Following bowel resection, partial obstructive effects may explain the small bowel enlargement in the jejunum. However, the significant increase in mucosal parameters in the remnant of ileum must be considered as an indirect measure of true structural intestinal adaptation. Therefore, many investigators examine the adaptation process only in the remaining ileum (10, 19). Although bowel and mucosal weight increased significantly in jejunum in our experiment, this increase was not associated with increased mucosal DNA, mucosal protein, and villous height in this segment. However, a marked increase in ileal bowel and mucosal weight was accompanied by

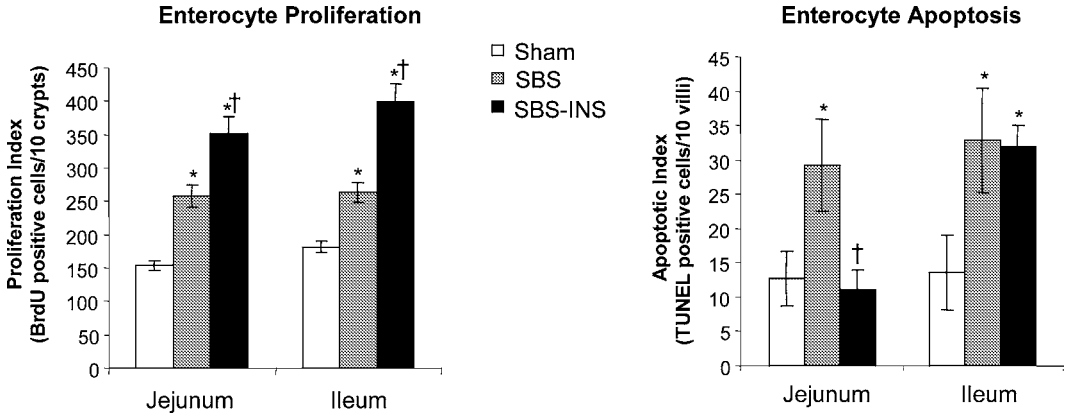

Fig 3. Effect of bowel resection and oral insulin (INS) on crypt cell proliferation and enterocyte apoptosis in jejunum and ileum (mean ± SE). 5-BrdU incorporation into proliferating jejunal and ileal crypt cells was detected with a goat anti-BrdU antibody, and TUNEL assay was used to determine enterocyte apoptosis. $*P < 0.05$, SBS (short bowel syndrome) vs sham rats; $\dagger P < 0.05$, SBS-INS vs SBS rats.

a twofold increase in mucosal DNA and protein, 15% increase in villous height, and 40% increase in crypt depth compared to SBS-untreated animals, suggesting an active proliferating process. As in our previous reports (16), apoptosis in remaining bowel segments increased significantly following massive small bowel resection. Increased enterocyte apoptosis has been described by many investigators in animal models of SBS and was considered as a mechanism that counterbalances the increased enterocyte proliferation in order to reach a new homeostatic point during intestinal adaptation (19). In addition, increased apoptosis promotes disposal of genetically aberrant stem cells and prevents tumorogenesis. Oral INS resulted in decreased enterocyte apoptosis in jejunum. Both increased cell proliferation and reduced cell apoptosis may be responsible for an increased enterocyte mass in the jejunum during adaptation. In the remaining ileum, oral INS administration led to a much more significant increase in enterocyte proliferation compared to the jejunum, without a change in enterocyte apoptosis. Increased enterocyte proliferation rather than apoptosis is responsible for the bulk of the enterocyte mass in the remaining ileum. Although the composition of the rodent chow regarding protein, fat, and carbohydrate may affect the adaptive response to intestinal resection, it seems highly unlikely that the small amount of additional protein (about 1 mg/day in our model) was responsible for the observed strong adaptive response without a specific INS action. Functional adaptation was not studied in the present experiment; however, an increased absorptive surface area is supposed to be accompanied by increased nutrient absorption. The effect of oral INS in decreasing weight loss after bowel resection is consistent with whole-body anabolic effects of this agent. Since adaptation is related to morbidity and mortality, we hope that the enhanced adaptation will decrease the death rate, but the limitations of our model prevent firm conclusions.

Although the pharmacokinetics of conventional INS given via the gastrointestinal route makes it virtually impossible to replicate the normal pattern of INS action, several studies have reported a hypoglycemic response to oral INS (20). In addition, the facts that INS is present in human and pig colostrum and mature milk and that INS receptors are present on both luminal and basolateral membranes of the enterocyte suggest its potential role in small intestinal growth and development (21). The evidence that oral INS has a trophic effect on intestinal mucosa comes from experiments by Shulman et al., who have demonstrated that oral INS stimulates an increase in ileal mass and disaccharidase activity (12) and increases ileal lactase activity (13) in the newborn miniature pig. In a recent clinical trial, Shulman showed that enteral administration of INS to preterm infants (26–29 weeks of gestational age) leads to higher lactase activity and less feed intolerance (22).

Extensive studies in various experimental models have established the positive effect of oral INS on the small intestinal mucosa. INS stimulates epithelial cell proliferation and differentiation of intestinal epithelial cells in vitro (23). INS accelerates enterocyte proliferation in the intestinal mucosa of suckling mice (24). INS increases maltase, lactase and aminopeptidase activities in villous cells and the concentration of the secretory component of immunoglobulins in crypt cells in rats (25). INS-receptor densities are selectively associated with intestinal mucosal growth in neonatal calves (26). In a recent study, we examined local and systemic effects of oral INS supplementation in rats in the postweaning period. We demonstrated that treatment with oral INS resulted in

mild hypoglycemia; however, levels of glucose were in the normal range. We have also shown that oral INS had a trophic gut effect that was evidenced by increased bowel circumference, mucosal weight, mucosal DNA, and villous height. The maximal changes were observed in the first week of INS treatment, and most significant changes were observed in the proximal intestine (27). The results of the present study add to the evidence that oral INS has a trophic effect on the remaining small bowel following massive small bowel resection. The observed effect of oral INS on proliferating mucosa in this model was much more significant than its effect on nonproliferating mucosa in our previous model (normal rats in the post-weaning period).

In conclusion, oral INS supplementation significantly enhanced adaptive gut growth in a rat model of SBS. The most significant changes were observed in the remaining ileum. The data on intestinal mass and cellularity suggest potential therapeutic roles of oral INS in enhancing intestinal adaptation following massive small bowel resection.

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