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Bombesin Improves Survival from Methotrexate-Induced Enterocolitis

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Objective

The authors determined whether bombesin could improve survival from methotrexate (MTX)-induced enterocolitis.

Summary Background Data

Bombesin prevents gut mucosal atrophy, which is produced by feeding rats an elemental diet. Administration of MTX produces a lethal enterocolitis in rats fed an elemental diet.

Methods

On treatment day 0, 60 rats were divided randomly into three groups and fed an elemental diet (Vivonex TEN, Sandoz, Minneapolis, MN) as the only source of nutrition. Groups were subdivided further to receive either saline or bombesin (10 μg/kg, subcutaneously, three times a day) beginning either on day 0 or day 14. Methotrexate (20 mg/kg, intraperitoneally) was given to all rats 14 days after the start of an elemental diet.

Results

Bombesin prevented the mucosal atrophy in the ileum produced by the elemental diet and significantly decreased mortality in rats given MTX (whether given as a pretreatment or at the time of MTX administration).

Conclusion

Bombesin significantly improved survival in a lethal model of MTX-induced enterocolitis, possibly by maintaining gut mucosal structure. Administration of bombesin to patients receiving chemotherapy may be clinically useful in preventing the severe enterocolitis induced by various chemotherapeutic agents.

Bombesin, a tetradecapeptide originally isolated from the skin of the frog *Bombina bombina,* is analogous to mammalian gastrin-releasing peptide.[1] Bombesin-like immunoreactivity is found widely distributed throughout the gastrointestinal tract of rats, guinea pigs, dogs, and humans.[1–5] Bombesin has multiple actions in the gastrointestinal tract, including stimulation of pancreatic, gastric, and intestinal secretion and release of all gut hormones except secretin.[1] In addition, bombesin, found in the milk of mammals,[6] stimulates growth of numerous gastrointestinal tissues. Lehy and colleagues[7,8] reported that bombesin, given orally or subcutaneously, stimulates the growth of gut and pancreas in neonatal rats, suggesting that bombesin may play an important role in early development of the gastrointestinal tract. In adult rats, bombesin stimulates the growth of the pancreas[9,10] and prevents mucosal atrophy of the small intestine, which is produced by a liquid elemental diet.[11]

Significant gut atrophy can increase susceptibility to intestinal injury.[12-14] Because of this, bombesin may be clinically useful to maintain gut mucosal structure during periods of injury (for example, trauma or administration of chemotherapy).

Chemotherapy is an important and standard armamentarium in the treatment of various cancers; however, the efficacy of these agents often is limited by severe side effects and toxic sequelae. In addition to their toxic effect on cancer cells, chemotherapeutic agents also affect normal tissues that have a high rate of proliferation, such as hematopoietic cells of the bone marrow and actively-dividing crypt cells of the gut mucosa. One of the toxic sequela of these agents (e.g., MTX) is intestinal injury and enterocolitis;[15-17] this side effect is compounded by atrophy of the gut mucosa. In experimental studies,[18-21] administration of MTX (20 mg/kg) to rats maintained on an elemental diet produced lethal enterocolitis. Fox and colleagues[20] demonstrated that a glutamine-supplemented elemental diet resulted in significant reduction of intestinal injury and translocation of indigenous gut bacteria, and, moreover, improved survival from this model of lethal enterocolitis.

Because bombesin is known to stimulate gut mucosal growth, we postulated that administration of bombesin could prevent the lethal enterocolitis associated with MTX. Therefore, the purpose of our study was twofold: 1) to examine the effect of bombesin on gut atrophy induced by a frequently-used elemental diet, Vivonex TEN (Sandoz, Minneapolis, MN), and 2) to determine whether bombesin could improve survival from a model of lethal MTX-induced enterocolitis.

METHODS

Experimental Design

In the first experiment (Fig. 1), 42 male Fischer 344 rats (240 to 260 g; Harlan Sprague-Dawley, Indianapolis, IN) were acclimated for 2 weeks at a constant temperature (22 C) with 12 hours of light and dark cycles and fed a standard rat chow (Ralston Purina, St. Louis, MO) *ad libitum*. After an overnight fast, six rats were killed (chow control). The abdomen of each was opened and the pancreas and small

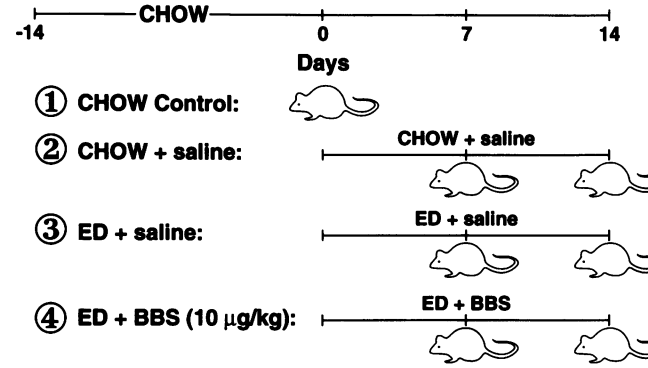

Figure 1. Experimental design for examination of bombesin effect on elemental diet-induced gut atrophy. Numbers note four groups as shown. Picture of rat denotes time of sacrifice for each group. ED = elemental diet; BBS = bombesin.

intestine (from the ligament of Treitz to the ileocecal junction) were removed. All intestinal segments were carefully trimmed of mesentery and suspended vertically with a 10-g weight to ensure constant lengths; two 20-cm segments of jejunum and ileum were analyzed (Fig. 2). Both segments were opened longitudinally and blotted dry, and the mucosa was scraped carefully from the underlying seromuscular layer using a glass slide as scraper. The mucosa, from the jejunum and ileum, and the pancreas were weighed and immediately frozen at −70 C until assayed for DNA and protein content. The remaining 36 rats were divided randomly into three groups of 12 rats each. The first group was continued on standard rat chow and injected with saline;

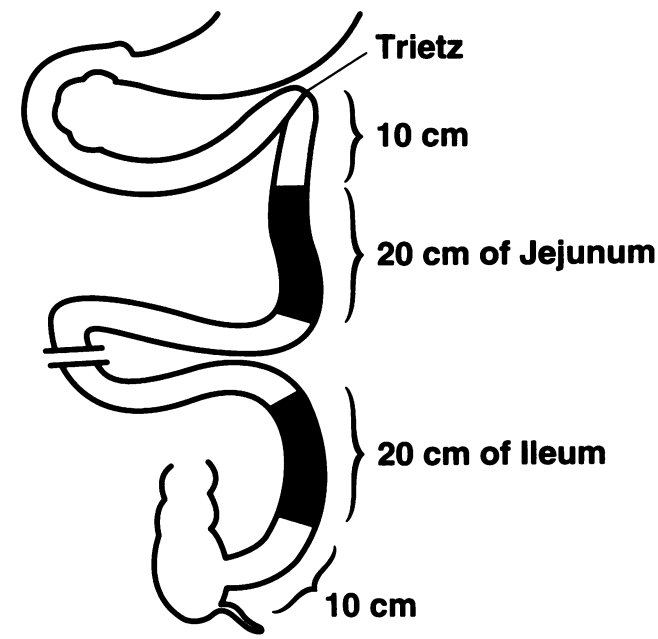

Figure 2. Schematic diagram of gastrointestinal tract illustrating two segments of small intestine taken for the study, shown by black segments.

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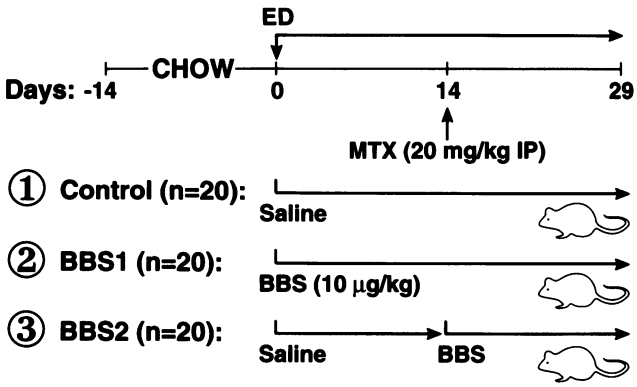

Figure 3. Experimental design for examination of bombesin effect on survival from MTX-induced enterocolitis. Numbers note three groups as shown. After MTX injection at day 14, rats were observed every 12 hours for mortality. BBS = bombesin; BBS1 = groups receiving bombesin on day 0; BBS2 = groups receiving bombesin on day 14.

the other two groups were started on an elemental diet (Vivonex TEN) and injected with either saline or bombesin (10 μg/kg, Bachem, Torrance, CA) mixed with gelatin subcutaneously, three times a day. The group that was fed chow and the elemental diet group, injected with bombesin, were isocalorically pair-fed to the elemental diet group injected with saline. On days 7 and 14, six rats from each of the three groups were killed by decapitation. The rats were weighed and killed beginning at 8:00 A.M.; this continued with one rat from each group until all were killed (approximately 1:00 P.M.). The pancreas and mucosa of both the jejunum and ileum were collected and weighed as described and frozen for DNA and protein measurement. In addition, 10-cm segments of proximal jejunum and distal ileum were fixed in buffered formalin, subsequently embedded in paraffin, and processed in a routine manner. Sections (5 μm) were stained with hematoxylin and eosin for light microscopic examination.

In the second experiment, 60 male Fischer 344 rats were weighed and randomly divided into three groups of 20 rats (Fig. 3). Rats were acclimated for 2 weeks on standard rat chow. After an overnight fast, all groups were started on the elemental diet (day 0). In addition, the groups of rats were subdivided further to receive either saline (control), bombesin starting on day 0 (BBS1), or bombesin starting on day 14 (BBS2) mixed with gelatin and administered subcutaneously three times a day. On day 14, all rats received a single injection of MTX (20 mg/kg, intraperitoneally) and were observed every 12 hours for mortality. The rats that died underwent necropsies immediately, and their small intestines were fixed and processed for histologic examination.

Peptide Preparation

A stock solution of bombesin was prepared by first dissolving the amount needed for the study in 1 mL of sterile water containing 0.1% (w/v) bovine serum albumin (Calbiochem-Behring, La Jolla, CA). This stock solution was diluted to the required concentration with saline containing 1% bovine serum albumin. Aliquots of 1% bovine serum albumin and bombesin (sufficient for injections of all rats of a given group for the day) were stored in plastic tubes at −20 C. To prolong the rate of absorption after each injection, bombesin in saline was mixed 1:4 (v/v) with 8% (w/v) hydrolyzed gelatin (Sigma Chemical Co., St. Louis, MO) before administration (final volume 0.5 mL).

DNA and Protein Measurement

Tissues were thawed and homogenized (Polytron, Kinematic GmbH, Kriens-Luzern, Switzerland). The DNA content was measured by the Burton modification[22] of the diphenylamine procedure, with calf thymus DNA used as the standard. Protein content was determined by the method of Lowry,[23] with bovine serum albumin as the standard.

Statistical Analysis

Pancreatic weight and mucosal weight of intestinal segments, as well as DNA and protein contents, were normalized by body weight. Values are expressed as the mean ± SEM and analyzed using analysis of variance for two-factor factorial experiment. The two factors were treatment (chow + saline, elemental diet + saline, and elemental diet + bombesin) and day (7 and 14). Fisher's least significant difference procedure was used for multiple comparisons. The survival data was analyzed by log rank and Wilcoxon tests. In all instances, a p value of < 0.05 was considered significant.

RESULTS

Bombesin Prevents Elemental Diet-Induced Ileal Atrophy

The body weight of the chow-fed group was significantly higher than that of the elemental diet-fed groups; however, there were no significant differences in either the initial or final body weight between elemental diet-fed groups at days 7 and 14 (data not shown).

Mucosal weight of the ileum from rats fed an elemental diet was decreased significantly by 22% at day 7 and by 31% at day 14 compared with rats given chow (Fig. 4). Similarly, total DNA content of ileal mucosa was decreased by 26% at day 7 and by 28% at day 14; total protein content was decreased by 22% at day 7 and by 23% at day 14. Bombesin treatment significantly reduced this mucosal atrophy; mucosal weight was increased by 15%

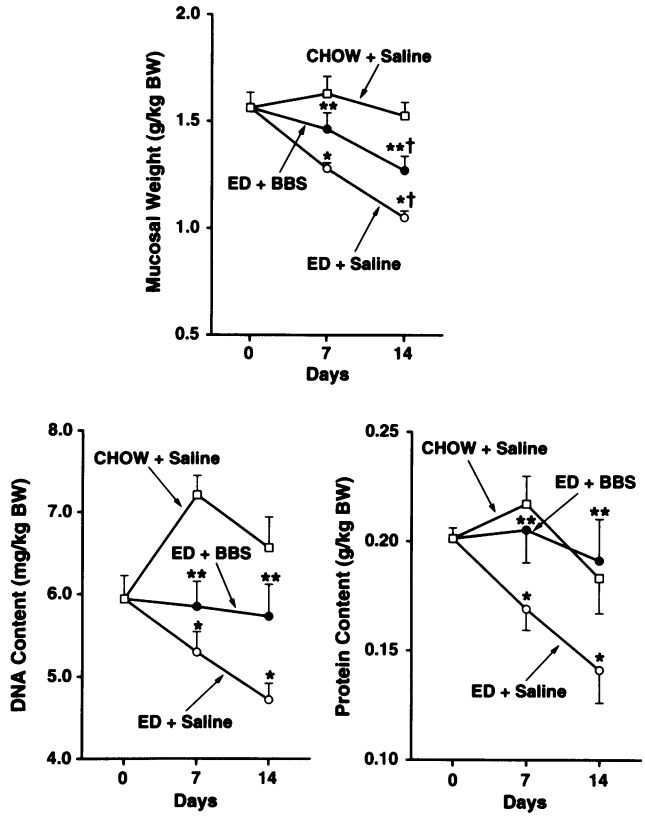

Figure 4. Mucosal weight, DNA, and protein contents of ileal segment corrected for body weight (N = 6, mean ± SEM, * = $p < 0.05$ *vs.* CHOW + Saline, ** = $p < 0.05$ vs elemental diet + Saline, † = $p < 0.05$ *vs.* same group from Day 7). ED = elemental diet; BBS = bombesin.

at day 7 and by 21% at day 14. Bombesin treatment increased total DNA content by 10% at day 7 and by 21% at day 14, and total protein content by 21% at day 7 and by 32% at day 14 compared with rats given an elemental diet alone (Fig. 4). Figure 5 shows representative histologic sections from the ileum of rats given chow, elemental diet alone, and elemental diet + bombesin; elemental diet produced a significant gut mucosal atrophy that was prevented by bombesin.

In contrast to the ileum, the elemental diet used in our study did not produce a mucosal atrophy in the jejunum (Fig. 6). The pancreas was removed and analyzed as an *in vivo* bioassay of bombesin activity and, as expected, bombesin stimulated pancreatic growth at days 7 and 14 (data not shown).

Bombesin Significantly Reduced MTX-Induced Mortality

Before MTX injection, there were no significant differences in the body weights among the three groups of rats given an elemental diet; however, after MTX injection, the body weight of the control group (saline-injected) was significantly decreased compared with the two groups that were injected with bombesin (data not shown). After MTX injections, rats became lethargic by day 3, had severe bloody diarrhea, and finally died, usually by day 10. At death, rats were immediately necropsied and found to have diffuse enterocolitis without evidence of perforation. Histologic examination of the entire intestine demonstrated diffuse vascular congestion and edema, sloughed epithelium, and bleeding into the lumen (Fig. 7). Some of the areas of the small intestine also were found to have villus tip necrosis and lymphocyte infiltration. Only 40% of rats given an elemental diet and saline injections survived after administration of single doses of MTX; however, in marked contrast, 90% to 100% of rats given bombesin, either starting on the day of MTX injection or 14 days before MTX, survived (Fig. 8).

DISCUSSION

We have shown that after feeding rats an elemental diet, bombesin reduces gut mucosal atrophy, confirming

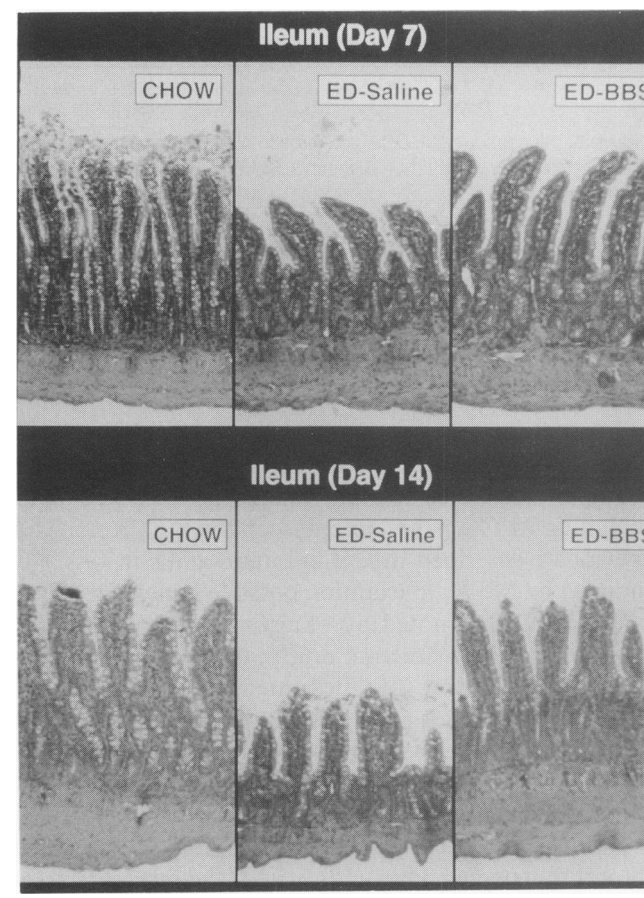

Figure 5. Histologic section of full thickness ileum from each group in same magnification. Elemental diet produced significant atrophy, and bombesin prevented this atrophy both at days 7 and 14. ED = elemental diet; BBS = bombesin.

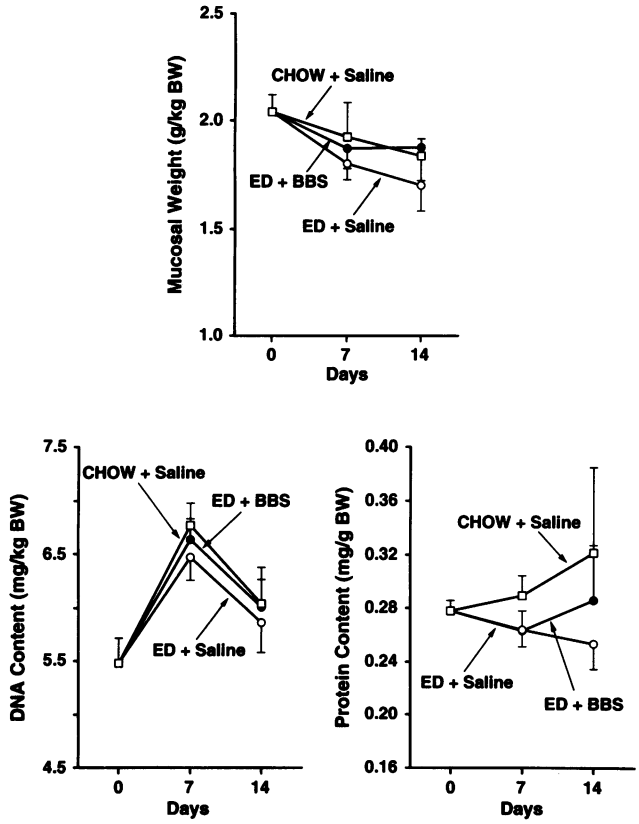

Figure 6. Mucosal weight, DNA, and protein contents of jejunal segment corrected for body weight (N = 6, mean ± SEM). ED - elemental diet; BBS = bombesin.

our previous findings.[11] In addition, we have demonstrated that bombesin significantly improves survival of rats in a model of severe enterocolitis induced by MTX.

Chemotherapy commonly produces structural and functional damage to the intestinal mucosa in cancer patients, a majority of whom already are malnourished.[13,14] A major side effect of many chemotherapeutic agents, including MTX, is severe enterocolitis.[15–17] We used a previously described model of enterocolitis in rats,[18–21] induced by MTX, to examine possible beneficial effects of bombesin. As noted by other investigators,[13] MTX produces a severe mucosal injury, demonstrated by denuded villi, bleeding into the lumen, and necrosis of the villi along the small intestine. Identifying an agent that prevents this severe mucosal injury may be clinically beneficial to cancer patients who require certain chemotherapeutic agents.

Bombesin, a hormone trophic to the gut, prevented the gut mucosal atrophy and reduced the mortality associated with the MTX-induced enterocolitis. Similarly, Fox and colleagues[20] demonstrated that the addition of glutamine—a nonessential amino acid that stimulates gut mucosal growth—to an elemental diet significantly improved survival in rats after MTX injection. Collectively, these studies suggest that the beneficial effects of both bombesin and glutamine are caused by their trophic effect on the gut mucosa. Therefore, maintenance of gut mucosal structure appears crucial to survival from the enterocolitis induced by MTX.

The mechanisms underlying the trophic effect of bombesin on gut mucosa are not known. A direct action of bombesin on gut mucosal growth is possible, but few (if any) bombesin-binding sites have been identified in the mucosal layer of the small intestine; rather, binding sites are located mainly in the muscle and submucosal layers.[2] Therefore, an indirect effect of bombesin is more likely. With the exception of secretin, bombesin stimulates the release of all gut hormones, including neurotensin and other trophic hormones.[1] Neurotensin has been shown to prevent gut atrophy induced by an elemental diet,[11] stimulate gut growth in rats fed a regular chow,[24]

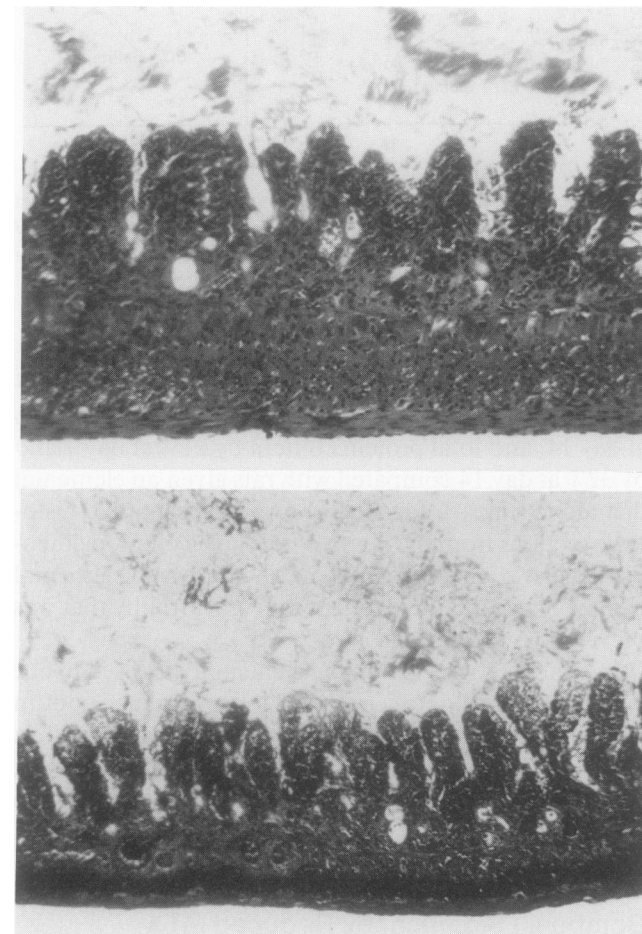

Figure 7. Histologic sections of MTX-induced intestinal injury. The top photograph illustrates vascular congestion, villi denuded of epithelium and bleeding into lumen, which were seen throughout the small intestine. The bottom photograph illustrates villus tip necrosis and lymphocyte infiltration, which occasionally were seen in small intestine.

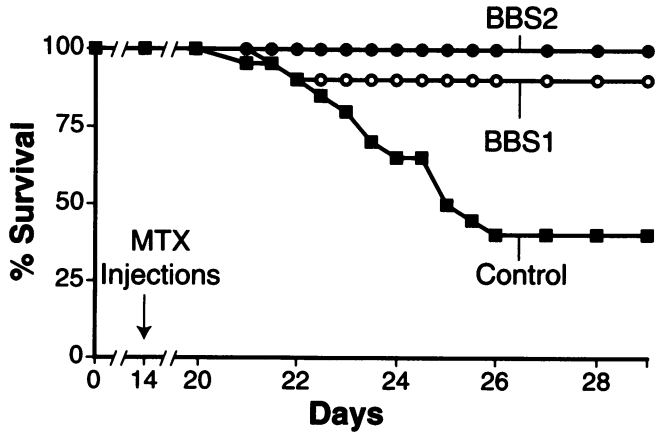

Figure 8. Survival curve from MTX-induced enterocolitis. Using log rank and Wilcoxon test, BBS1, and BBS2 are shown to have significantly greater survival after MTX injection.

and augment intestinal regeneration after small bowel resection.[25] Therefore, bombesin may produce its trophic effect on the gut via the release of neurotensin. Other possible mechanisms include an indirect effect through stimulation of pancreaticobiliary secretions, which are known to stimulate mucosal growth.[26,27]

Another possible beneficial effect of bombesin that may play an important role in reducing mortality after MTX could be caused by a stimulatory effect of bombesin on the immune system. Bombesin is known to stimulate the growth of mature T cells,[28] increase intestinal secretion of immunoglobulin A,[29] and enhance natural killer activity of both peripheral blood mononuclear cells[30] and intestinal lamina propria mononuclear cells.[31] Intestinal injury can lead to increased permeability and translocation of enteric gut bacteria.[12,32-34] Although not specifically examined in this study, bacterial translocation is a likely event after MTX injection that may play a significant role in the death of these rats; bombesin may reduce mortality by enhancing immune function. In fact, Haskel and colleagues[12] demonstrated recently that administration of bombesin significantly decreased bacterial translocation in rats given an elemental diet.

Our study demonstrates the protective trophic effect of bombesin on gut mucosal epithelium. Bombesin reduced gut atrophy produced by an elemental diet and improved survival from an intestinal injury model of MTX-induced enterocolitis, whether given as a pretreatment or at the time of injury. Bombesin may be of therapeutic use in preventing enterocolitis in patients receiving toxic, chemotherapeutic agents. Significant improvement from the side effects of chemotherapeutic agents, such as enterocolitis, can lead to better tolerance of these agents and more efficient therapy for oncology patients.

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Discussion

DR. JOHN DELANEY (Minneapolis, Minnesota): This is a very nice paper with potential clinical usefulness. I was asked to discuss it because I have had an interest in radiation injury and its prevention in the small bowel. Methotrexate and radiation have similarities. Both inhibit Crypt cell replication and therefore restitution of the mucosa.

The small bowel has some unique characteristics that are difficult to interpret. One of those characteristics is extremely rapid replacement of the mucosa, every 2 days in the rat. In addition, the intestine contains myriad bacteria which can elaborate toxins that get into the circulation when the bowel is injured. When you perturb one variable in the small bowel, everything else is perturbed as well. It is incidentally a huge reservoir of lymphoid tissue, as the transplanters have found out. The gut is also the largest endocrine organ in the body. So anything that you disturb changes numerous other variables. That is what makes this experiment difficult to explain.

For example, giving the bombesin at time of the methotrexate injury had the same effect as when it was given prior to the injury. Either the bombesin had to block the effects on the enterocytes or it caused a tremendous acceleration of enterocyte replication following the injury. I would be interested in speculations from the authors.

Methotrexate has multiple actions, one of which is to inhibit bone marrow and induce leukopenia. We know that leukopenia itself can lead to enterocolitis. I would ask the authors if they studied hematologic parameters and were they influenced by bombesin? They mentioned the possibility of bacterial translocation; was that studied that as a possible cause of death?

We heard in an earlier paper at this meeting that glutamine had some influence on replication of tumor cells. Glutamine is a trophic factor for the small bowel, like bombesin. Jim Thompson's lab has had an interest in cancer trophic factors. I would ask if they have delved into the possibility that bombesin has trophic effects on malignancies? In other words, this agent cannot be used clinically to protect the intestine until we are confident that the bombesin does not interfere with the desired effects of the methotrexate on the target tumor.

DR. KENNETH R. SIRINEK (San Antonio, Texas): I would like to congratulate Dr. Chu on his presentation and to thank the authors for providing a copy of their manuscript for my review prior to the meeting.

This study represents another contribution by my University of Texas colleagues at Galveston for the understanding of the physiology, pathophysiology, and now possible clinical applications of gut peptides. I would like to ask two questions and to disagree with one of their conclusions.

Are the authors aware of any effect of bombesin on the absorption of an elemental diet? And secondly, why was the jejunum protected, and the only site of enterocolitis was the ileum?

I thoroughly agree that they have shown a decreased mortality from the methotrexate-induced enterocolitis by the administration of bombesin. However, I disagree with their conclusion that the beneficial effect occurred by preventing gut mucosal atrophy.

Group 3 rats did not get bombesin until day 14, the same day that the methotrexate was given. Mucosa in this group of animals should have atrophied during that 14-day period just like the control group. For this group of animals it would appear more plausible that bombesin may have acted as a competitive inhibitor of methotrexate-induced enterocolitis. This could be the same receptor that is responsible for stimulating cell growth or for its destruction.

Could this hypothesis of competitive inhibition be demonstrated by giving additional doses of methotrexate to displace the bombesin from this receptor site?

DR. ARTHUR H. AUFSES, JR. (New York, New York): I would also like to congratulate Dr. Chu and his colleagues on a very interesting study which, as Dr. Delaney pointed out, may have very significant clinical implications.

I notice, however, that although they describe this as a model of enterocolitis, the only slides that we saw were of the jejunum and ilium. And I wonder what the changes actually are in the colon.