Proglumide, A Gastrin Receptor Antagonist, Inhibits Growth of Colon Cancer and Enhances Survival in Mice

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Some tumors are responsive to hormone manipulation. Some gastric and colonic adenocarcinomas from both humans and animals have specific gastrin receptors. A transplantable mouse colon adenocarcinoma cell line (MC-26) contains gastrin receptors; growth of MC-26 colon cancer *in vivo* is stimulated by pentagastrin (PG). The purpose of this study was to determine whether a gastrin-receptor antagonist, proglumide (PGL), would inhibit growth of MC-26 colon cancer and prolong survival in tumor-bearing mice. Subcutaneous tumors were induced by injecting single-cell suspensions of MC-26 cells into 50 mice divided into 10/group. In Experiment 1, all mice received 1×10^5 tumor cells and treatment groups were divided as follows: Group A received intraperitoneal (IP) saline (0.2 ml tid beginning on day 1); B, IP, PGL (250 mg/kg tid) from day of tumor cell inoculation; and C, IP PGL (250 mg/kg tid) from day 7 after tumor implantation. In Experiment 2, mice were inoculated with half the number of tumor cells. Group I mice received saline and Group II received PGL in the same manner starting on day 1. Tumors were measured and all mice were sacrificed on day 23. In Experiment 1, mean tumor area in Group B (PGL-treated) was significantly smaller than Group A on days 11, 14, 17, and 21. Tumors of Group C were significantly smaller than controls on day 21. Survival of PGL-treated mice was significantly prolonged. In Experiment 2, mean tumor area, mean tumor weight, and tumor DNA and RNA content were significantly less in the PGL-treated group than control. It was concluded that growth of a gastrin-responsive colon cancer was inhibited and host survival was enhanced by treatment with a gastrin-receptor antagonist. Hormone manipulation may be a useful treatment for gastrointestinal cancers.

SOME CANCERS (*i.e.*, breast, prostate, and thyroid) may be successfully treated by hormone manipulation. Hormone therapy confers definite palliation in a certain proportion of patients with these tumors. The gastrointestinal hormones, gastrin, cholecystokinin, and secretin, stimulate the growth of normal gut mucosa[1] and pancreas[2,3] of rats, mice, and hamsters. We have been interested in the interactions of these trophic gastrointestinal hormones and gastrointestinal tumors.

Colon cancer is second only to lung cancer as a cause of cancer death in the United States.[4] Treatment of colon cancer relies heavily on surgical resection. While multidisciplinary approaches to therapy have proven beneficial for many types of cancer, there has been no widely effective adjuvant therapy developed for the treatment of adenocarcinoma of the colon.

The hormone gastrin (produced by antral G-cells) has a trophic effect on portions of the alimentary tract of rats[1] and mice.[5] Our laboratory studies have shown that human gastric and colonic mucosa and adenocarcinomas of the stomach and colon contain specific receptors for gastrin.[6,7] The functional significance of these receptors is not yet clear.

We have developed a mouse colon adenocarcinoma cell line (MC-26) in tissue culture. When MC-26 cells are injected into Balb/c mice, tumors are produced in a dose-dependent manner. Winsett and colleagues[5] demonstrated that growth of the MC-26 colon cancer *in vivo* is stimulated by pentagastrin. Singh and associates[8,9] have found that MC-26 cells in tissue culture and MC-26 tumors growing in mice possess specific gastrin receptors.

Proglumide (Rotta Research Laboratories, Monza, Italy) is a derivative of glutamic acid[10] with a molecular weight of 334; it specifically and competitively inhibits the effects and the receptor-binding of gastrin[11] and the closely related peptide, cholecystokinin.[12]

Proglumide appears to be a safe drug which is remarkably free of adverse effects and has been used in the treatment of human peptic ulcer disease in Europe for approximately 10 years.[13,14] Lamers and Jansen[14] showed a 13% to 62% inhibition of gastric acid secretion by proglumide in patients with the Zollinger-Ellison syndrome.

The purpose of this study was to determine whether

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TABLE 1. *Experiment 1: Proglumide's Effect on Tumor Growth*

	N	Tumor Area (mm^2) Day 11	Day 14	Day 17	Day 21
Group A	9	9.2 ± 2.8	22.7 ± 3.9	54.6 ± 9.8	124.7 ± 10.8
Group B	10	2.4 ± 1.8*	4.8 ± 2.3*	17.0 ± 5.4*	57.6 ± 8.0*
Per cent		26%	21%	31%	46%
Group C	10	6.6 ± 2.5	17.3 ± 4.2	36.8 ± 8.9	78.3 ± 17.1*
Per cent		71%	76%	67%	63%

Tumor size = product of diameter 1 × diameter 2 = mm^2. Group A = saline-treated controls; Group B = treated with proglumide (250 mg/kg tid); Group C = proglumide treatment starting day 7 after tumor injection.

All mice received 1×10^5 MC-26 cells subcutaneously on day 1.

* $p < 0.05$ *versus* control.

proglumide will inhibit growth of the gastrin-responsive MC-26 colon carcinoma and whether treatment with proglumide will prolong survival in tumor-bearing mice.

Materials and Methods

The MC-26 tissue culture cells were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS). Cells used for experimental purposes were taken from passages 20 to 25 only to reduce tumor variability. For injection, the cells were harvested by trypsinization, washed in medium with 10% FCS, centrifuged and resuspended in medium without serum. Cell numbers were determined with a Coulter counter, and a final dilution was made so that the desired number of cells to be injected was contained in 0.2 ml of medium. The single-cell suspensions were injected subcutaneously into the interscapular region of male Balb/c mice.

Two separate experiments were performed *in vivo*. In Experiment 1, 30 Balb/c mice (20–25 g) were inoculated subcutaneously with 1×10^5 MC-26 cells; the mice were then randomized into three groups of 10 each. Group A was treated with saline (0.2 ml) three times daily; Group B received proglumide (250 mg/kg tid) beginning on the day of tumor inoculation; and Group C received proglumide (250 mg/kg tid) beginning 7 days after tumor implantation. Both saline and proglumide were given by intraperitoneal (IP) injection. One mouse in Group A died on day 5 from a traumatic injection and was excluded from the study.

We assessed growth of the tumor by measuring the two greatest perpendicular tumor diameters which were measured using calipers twice weekly. The tumor area was then calculated as the product of these diameters and expressed in mm^2. All measurements were performed by an observer who did not know whether the mouse belonged to a test or to a control group. Treatment and measurements were continued for 21 days after cell injection and then stopped. The mice were observed several times daily, thereafter, to determine mortality rates.

In Experiment 2, 20 mice were inoculated in a similar manner with half the number of MC-26 cells (5×10^4). The mice were then randomized into two groups. Group I received saline (0.2 ml tid), and Group II received proglumide (250 mg/kg tid); both were given from the day of tumor cell inoculation by IP injection. The tumor size was measured and the area calculated as described above. On day 23, all of the mice were sacrificed. Tumor, gastric fundus, pancreas, and colon (from ileocecal valve to rectum) were excised, rinsed in ice-cold saline, blotted dry, and weighed. These tissues were promptly frozen in liquid nitrogen until they were extracted for determination of DNA, RNA, and protein content.

Tissues were extracted for measurement of DNA, RNA, and protein content by means of a modification of the procedure of Ogur and Rosen.[15] DNA was measured by the Burton[16] modification of the diphenylamine method, after extraction into 0.5 N perchloric acid for 20 minutes at 90 C. DNA from calf thymus was used as standard. RNA was measured by the orcinol procedure using yeast RNA as standard.[17] Protein was determined by the method of Lowry and colleagues,[18] using bovine serum albumin as standard.

Statistical analysis of tumor size, body weight, organ size, DNA, RNA, and protein content was performed using the one-way analysis of variance (Anova) and by the Student's t-test. Survival data were analyzed by Gehan's generalized Wilcoxon test.[19] Significant differences are noted as $p < 0.05$.

Results

In Experiment 1, 100% of the mice developed palpable tumors. Mean body weights between groups were not significantly different throughout the experiment. By day 11, significant differences were noted when comparing tumor size (mm^2) of treated mice with the controls (Table 1, Fig. 1). Group B had significantly smaller tumors at all points of analysis from day 11 through day 21. Group C also had smaller tumors than the control group, but this difference did not become significant until day 21. By day 21, the tumor size of Group B was 46% of control ($p < 0.05$) and the tumor size of Group C was 63% of control ($p < 0.05$).

In addition to the effect on tumor growth, proglumide therapy also prolonged the survival of tumor-bearing mice (Fig. 2). The mean survival of Group A mice (controls) was 25.3 days (median 23 days). For Group B, the mean survival was 39.2 days (median 30.5 days). For Group C, mean survival was 36.6 days (median 28.5 days). Survival in both treated groups (B and C) was significantly longer ($p < 0.05$) than control, although all of the mice eventually succumbed to their tumors. By day 35, all of the control mice were dead, whereas 25% of the treated mice were

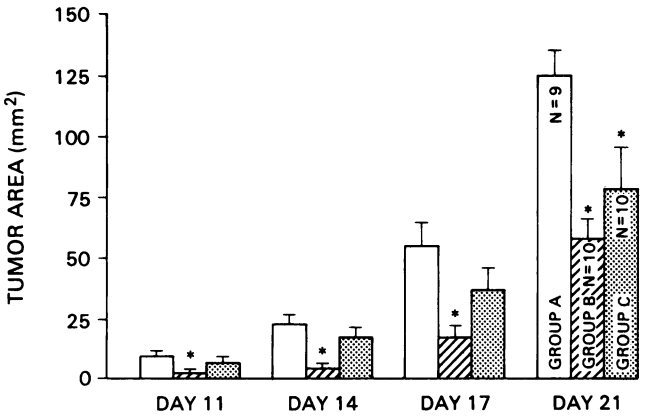

FIG. 1. Effect of different treatment regimens on tumor size in Experiment 1. Mice received 1×10^5 tumor cells subcutaneously on day 1. Group A, saline-treated; Group B, treated with proglumide [250 mg/kg tid] from day 1; Group C, treated with proglumide [250 mg/kg tid] from day 7. (* = $p < 0.05$).

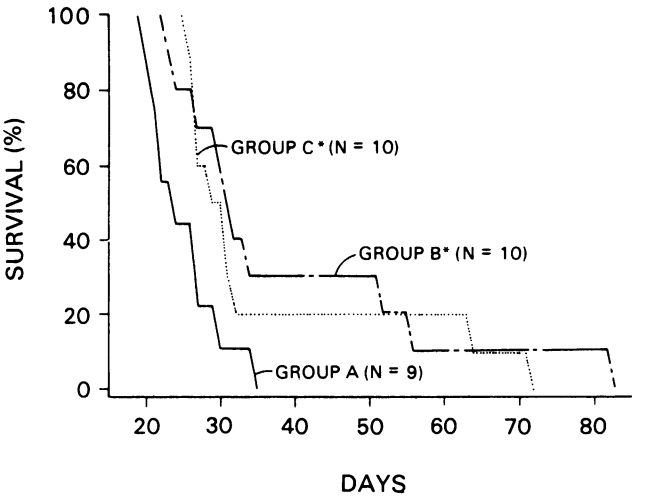

FIG. 2. Survival of mice in each group in Experiment 1. (* = $p < 0.05$)

still living. The last mouse in Group C died on day 72 and the last mouse in Group B died on day 83.

In Experiment 2, treatment was begun with either saline (Group I) or with proglumide (250 mg/kg tid) (Group II) on the day of tumor cell injection. A smaller number of MC-26 tumor cells was given to mice in this experiment so that early mortality would not interfere with our observations. As we found in Experiment 1, mean tumor area was significantly smaller in the proglumide-treated mice compared to controls (Fig. 3). All mice were sacrificed on day 23; seven of the 10 mice in Group I (saline) and nine of the 10 mice in Group II (PGL) had developed subcutaneous tumors. The mean body weights of the mice in each group were not significantly different. On the other hand, the mean weight of the tumors of the proglumide-treated mice was significantly smaller than control animals (Table 2).

We also analyzed tissues from the gastrointestinal tract of proglumide-treated and control mice to determine whether proglumide affected these normal tissues. There was a small but significant difference in the mean colon weights of proglumide-treated mice (237.2 mg) compared with controls (275.8 mg) ($p < 0.05$). There was no significant difference in the mean weight of the fundus or pancreas between the groups.

Excised tissues were analyzed for DNA, RNA, and protein content (Table 2). Tumors from the proglumide-treated mice contained significantly less DNA and RNA. The colons in the proglumide-treated mice had less RNA and protein content than controls ($p < 0.05$). Fundus DNA content was significantly less in proglumide-treated mice than in controls, but RNA and protein content were not different; this finding is consistent with the lack of difference between fundic weights. Pancreatic weight, DNA, RNA, and protein contents were not affected by proglumide treatment.

Discussion

Palliative treatment of metastatic breast cancer by oophorectomy was initially reported by Beatson in 1896.[20,21] Huggins and associates[22,23] in 1941 described orchiectomy and estrogen therapy for treatment of patients with advanced prostate carcinoma. Huggins and Bergenstal[24] subsequently reported the successful palliation of advanced prostate and breast cancer by adrenalectomy.

These surgeons were aware of stimulatory hormonal influences on normal target organs in which these tumors arose; they theorized that tumors arising from these hormone-responsive organs might be stimulated by the same

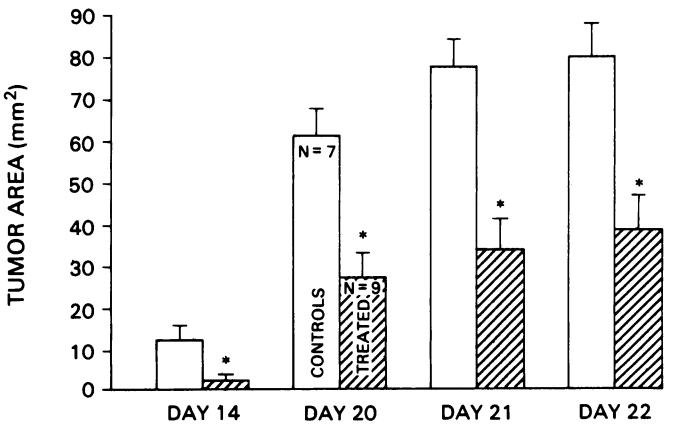

FIG. 3. Tumor size of saline-treated controls compared to proglumide treated (250 mg/kg tid) beginning on the day of inoculation of 5×10^4 tumor cells. In Experiment 2, mice received 5×10^4 MC-26 cells subcutaneously on day 1. (* = $p < 0.05$)

TABLE 2. *Experiment 2: Proglumide's Effects on Tissue Weight and Composition*

	Tumor	Colon	Fundus	Pancreas
Group I (saline)				
Tissue weight (mg)	332.5 ± 36.5	275.8 ± 9.9	103.4 ± 4.8	319.1 ± 15.4
DNA (μg)	852.4 ± 81.7	875.9 ± 52.4	408.3 ± 18.7	696.1 ± 32.2
RNA (mg)	4.35 ± 0.4	3.2 ± 0.2	1.19 ± 0.04	10.7 ± 0.5
Protein (mg)	38.5 ± 4.6	25.5 ± 1.3	5.7 ± 0.5	45.6 ± 2.3
Group II (PGL)				
Tissue weight (mg)	173.5 ± 40.7*	237.2 ± 7.0*	98.9 ± 2.3	318.3 ± 17.2
DNA (μg)	380.3 ± 51.2*	770.2 ± 34.1	302.7 ± 8.0*	685.3 ± 34.3
RNA (mg)	2.57 ± 0.5*	2.6 ± 0.1*	1.24 ± 0.1	11.0 ± 0.8
Protein (mg)	22.4 ± 5.6	21.5 ± 1.1*	4.9 ± 0.4	43.7 ± 3.1

* = $p < 0.05$ compared to controls.

N = 10 animals per group except for tumor data where only tumor-bearing animals were included with N = 7 for Group I and N = 9 for Group II.

Group I, saline; Group II, proglumide-treated. There was no significant difference in tumor incidence between the groups.

hormones. Their goal was to retard tumor growth by administration of antagonistic hormones or by ablation of the sources of stimulatory hormones. Since these landmark studies were reported, the treatment of certain cancers (*i.e.* breast, prostate, and thyroid) by hormone manipulation (either by addition of hormones, antihormones, or by ablation of hormone-producing organs) has become firmly established. We have been interested in determining whether cancers of the gastrointestinal tract could be similarly treated by hormone manipulation.

Johnson and colleagues[25] demonstrated in 1969 that gastrin stimulates protein synthesis in gastric and duodenal mucosa of rats. Gastrin has a trophic effect on the mucosa of the gastric fundus, duodenum, colon, and the pancreas.[1] Antrectomy in rats causes atrophy of mucosa of the gastric fundus, duodenum, colon, and the pancreas. Pentagastrin treatment of rats after antrectomy prevents atrophy of the gut and pancreas.[26]

Peptide hormones have trophic effects on normal tissues of the gastrointestinal tract. Can the growth of tumors of the gastrointestinal tract be influenced by hormonal manipulation? Townsend and colleagues[27] found that caerulein (a natural analog of CCK) combined with secretin stimulated growth of H2T pancreatic ductal adenocarcinoma implanted in the hamster cheek pouch. Winsett and colleagues[5] demonstrated that pentagastrin administration stimulated growth of MC-26 mouse colon adenocarcinoma and that mice with gastrin-stimulated tumors died at a significantly faster rate than controls. We have found that MC-26 mouse colon adenocarcinoma,[8,9] as well as some human gastric and colonic adenocarcinomas,[6,7] contain gastrin receptors.

Proglumide is a specific, competitive inhibitor of gastrin[11] and of cholecystokinin,[12] a peptide hormone closely related to gastrin.[28] Proglumide inhibits the action of gastrin and cholecystokinin apparently by inhibiting the binding of these two hormones to their respective cell membrane receptors.[11,12] Johnson and Guthrie[29] found that proglumide inhibits the pentagastrin-stimulated increase in DNA synthesis in the rat fundic, duodenal, and colonic mucosa and pancreas. Since MC-26 colon cancer is stimulated by pentagastrin, and since proglumide inhibits pentagastrin stimulation of normal colonic mucosal growth, we wanted to know if proglumide would affect growth of MC-26 colon cancers.

In the experiments reported here, mice received no exogenous gastrin; tumor growth was delayed and survival was enhanced by proglumide treatment of mice with MC-26 colon cancer. This is evidence that endogenous gastrin stimulates growth of the MC-26 colon cancer. The findings in Experiment 1 show that proglumide not only inhibits the growth of MC-26 colon cancer when administration was begun on the day of cell injection (Group B), but also inhibits the growth of established colon cancer (Group C). Mice in Group C had treatment begun 7 days after tumor cell injection and received treatment for only 14 days. This is in contrast to mice in Group B, which received 21 days of treatment. Proglumide prolonged survival in tumor-bearing mice when it was begun on the day of tumor cell injection and when it was begun when the tumors were established (7 days after tumor cell injection).

Measured tumor area was smaller in the proglumide-treated mice in Experiment 2. When the tumors were excised, the mean tumor weight and DNA and RNA content were significantly lower in the proglumide-treated group.

Despite the fact that these were short-term studies (14 or 21 days of treatment), there were clearly persistent effects of proglumide treatment on tumor size and survival, even when the onset of treatment was delayed. Although all of the proglumide-treated mice in Experiment 1 eventually died, only one dose of proglumide was tested. It is possible that higher doses of proglumide may produce even greater retardation of tumor growth and prolongation of survival. The effects of proglumide treatment may be

enhanced by combination therapy with cancer chemotherapeutic agents.

We cannot determine the mechanism of the proglumide antitumor effect from these studies. Our results may be explained either by direct effects of proglumide on proliferation of tumor cells, or by interference with endogenous gastrin. *In vivo* and *in vitro* studies are now under way to answer these questions.

Growth of normal mucosa of the colon and gastric fundus can be stimulated by gastrin.[1,5] Proglumide treatment of mice with MC-26 colon cancer should block the effects of endogenous gastrin on the normal mucosa as well as inhibit tumor growth. In addition to the inhibitory effect on tumor growth, we observed an effect of proglumide treatment on the gastric fundus and the colon. DNA content of the gastric fundus was decreased and RNA and protein content were decreased in the colon. These findings provide further evidence that endogenous gastrin is important for maintenance of cell proliferation of normal gastrointestinal mucosa.

Surgical excision remains the only effective treatment for colorectal carcinoma. An effective treatment for advanced colon carcinoma has not been developed; there is no current widely effective systemic adjuvant therapy (which can be employed after resection) that can prolong survival of patients with colon cancer. Our study has shown that treatment with the anti-gastrin compound, proglumide, inhibits growth of the gastrin-responsive MC-26 colon cancer and prolongs survival in mice with MC-26 colon cancers.

The growth of some breast cancers can be inhibited by hormone manipulation. Identification of patients who have hormone-dependent breast cancers can be accomplished by analysis of breast cancers for the presence of estrogen and progesterone receptors. Patients whose breast cancers possess abundant amounts of estrogen and progesterone receptors respond to treatment with hormones, anti-hormones, or by ablation of hormone-producing organs; those patients whose tumors possess few or no hormone receptors do not respond to hormone manipulation.

We have now developed methods[6–9] by which we can detect and quantify gastrin receptors in normal gastric and colonic mucosa as well as cancers arising from these organs in mice and humans. Some breast carcinomas do not possess estrogen and progesterone receptors; similarly, gastrin receptors are not detectable in all gastric and colonic cancers. Analysis of gastrointestinal tumors for gastrointestinal hormone receptors may allow us to select those patients with gastrointestinal cancers who would respond to treatment with antihormones or hormone ablation. It may be possible in the future to develop therapeutic strategies for patients with gastrointestinal cancers which are based on manipulation of the effects of gastrointestinal hormones in a manner similar to current strategies which are successfully employed in the treatment of patients with breast cancer.

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Discussion

DR. R. SCOTT JONES (Charlottesville, Virginia): Dr. Beauchamp has presented fascinating data that may provide a new avenue or insight into increasing the understanding of the pathogenesis of colorectal cancer and perhaps a new strategy for its treatment. This is most interesting work, and he has presented the background information concerning receptor sites in the tumors and the influence of gastrin and gastrin analogues on growth of the tumors and survival of the animals.

In reflecting on the presentation and reviewing the previous material, several questions arise. One question is: What is the relation between the mouse tumor cell type and human colon cancer? Most human alimentary cancers are derived from mucus glands, and I am wondering whether the MC-26 cell is derived from mucus glands or these mucus cells or from other cells in the colon epithelium.

Second, what is the antitumor specificity of proglumide? Although the evidence is presented that there are gastrin receptors and that these are affected by gastrin, that does not necessarily mean that that is the mechanism by which this agent works. It is possible that this could be a nonspecific effect, and my question is: Does proglumide affect other tumor systems? Would they affect the growth of sarcomas or other tumors?

Thirdly, does proglumide have side effects when given *in vivo?* Most of the animals in this particular study had tumors, and it is not clear that we could separate the effect from the tumor and the drug. What happens if you give normal animals proglumide in doses employed in this study?

Lastly, since gastrin and CCK act on the same receptor, I would like to ask the authors to comment on the relative importance of gastrin *versus* CCK as a stimulator of colon mucosa or colon neoplasma.

I think this is a fine study, and it opens a unique area for the investigation of colon cancer.

DR. RICHARD E. WILSON (Boston, Massachusetts): This is obviously a fascinating presentation and provokes many questions, some of which Dr. Jones has already asked.

I would like to pose a couple more. First of all, this is a "false" system, injecting these tumor cells in the flank. There are many models for induction of GI cancers in the colon with DMH or other carcinogens. I wonder if the authors have attempted to see whether *in situ, in vivo* cancers in the normal position have been altered by the administration of proglumide.

Secondly, there are no animals cured in this system that they described. In other words, there is only a delay in mortality, as the life table plot for the first experiment showed. Have they been able to identify a dose with which, in fact, the cancer can be totally eradicated when therapy is given?

And, thirdly, do these tumors in the flank actually grow faster or larger when gastrin is given? In other words, is there proof in this model that gastrin makes the tumors more lethal?

DR. BERNARD M. JAFFE (Brooklyn, New York): I, too, would like to congratulate the authors on an elegant study and for the exciting potential that their technology provides for the treatment of patients with colon carcinoma.

I think there is no longer any question that gastrin is trophic to the colonic and gastric mucosa, malignant as well as benign. Nonetheless, despite the clear demonstration of gastrin receptors on these cancer cells by Drs. Thompson, Townsend, and their colleagues, the mechanism of this inhibitory action of proglumide is far from clear. In their manuscript, which I was privileged to preview, the authors suggest a direct effect of proglumide and inhibition of endogenous gastrin as two possible mechanisms of action of this inhibition. I would like to add a third possible mechanism, modulation of the immune response.

Because of the close localization of intraepithelial colonic lymphocytes adjacent to the peptide secreting cells of the gut, Drs. Perisic, McMillan, and associates in our laboratory have recently been studying the possibility that the GI peptides modulate the immune response. Vasoactive intestine peptide receptors on lymphocytes have previously been demonstrated, so there is at least some precedence for this approach. So far our studies have shown that gastrin is clearly suppressive at least for ConA- and for PHA-induced proliferation of mouse splenocytes. I would like to ask the authors to comment on the possible role of immunomodulation in their *in vivo* studies.

If, in fact, the response to proglumide is due to binding to the gastrin receptors on these tumor cells, I would also like to ask the authors to comment on the final mediator of the inhibition of the cell killing. Is it mediated by cyclic nucleotides, calcium, prostaglandins, or possibly another particular trophic factor?

DR. SAMUEL A. WELLS, JR. (St. Louis, Missouri): I would like to know if you have looked at a nude mouse system using human tumors. It seems that this would be a fairly relevant way to gain some insight into whether or not proglumide would be useful in the management of colorectal malignancies in man.

In some patients with carcinoma of the breast, the administration of antiestrogens is often associated with a flare reaction, where there is stimulation of tumor growth. Was a similar phenomenon noted in any of the experimental animals?

Is anything known about the mechanism of action of proglumide? You often find that there is a very slight difference in molecular structure between protagonist and antagonist, and although both pentagastrin and proglumide appear to compete for a similar receptor on the colon cancer cell, they cause different effects. Is it known why?

DR. STANLEY R. FRIESEN (Kansas City, Kansas): I am interested in the fact that gastrin normally would be expected to stimulate only normal parietal cells because traditionally that is where the gastrin end organ cell with its receptor is located. But to have it affect receptors on exocrine tumor cells as well is unexpected; perhaps, there is some modulator effect of the gastrin through another substance.

I think it would be interesting to ask whether that is possible.

The second question I have is: Since other polypeptides do have a trophic effect like gastrin's effect on parietal cells and secretin's and CCK's effect on their normal end organ cells, such as the pancreatic duct cells and pancreatic acinar cells, respectively, is there any evidence to show that secretin and CCK have any effect on tumor cells as is being implied by this paper today?