SUBSTANCE P INCREASES IN VITRO LYMPHOKINE-ACTIVATED-
KILLER (LAK) CELL CYTOTOXICITY AGAINST FRESH COLORECTAL
CANCER CELLS.

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November 1992

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements of the
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ABSTRACT

Gut associated lymphoid cells are modulated by gut hormones. We postulated that lymphokine-associated-killer (LAK) cell cytotoxicity of lymphocytes from the gut mucosa may be increased by substance P (SP).

Intestinal lamina propria mononuclear cells (LPMC) and colonic cancer cells were isolated from operative specimens by mechanical and enzymatic dissociation methods. Effector LAK cells were induced by culturing LPMC with 250 u/ml of recombinant interleukin-2 (IL-2). Substance P (10^{-5} M) was added to the culture. Targets consisted of fresh colon cancer cells, HT-29 and control cell lines. After 4 days of incubation, cytotoxicity was measured using a 4-hour ^{51}Cr release assay.

LAK cells showed moderate cytotoxicity against HT-29 and none against fresh colon cancer cells. LAK cells + substance P showed moderate cytotoxicity against HT-29 and strong cytotoxicity against fresh cancer cells. Cytotoxicity $\pm$ SEM at various effector-to-target (E:T) ratios was: (* denotes $p < 0.05$ compared with above).

Effector	Target	N	5:1	10:1	25:1	50:1
LAK	HT-29	7	2.8 ± 0.5	11.6 ± 1.0	12.3 ± 2.3	17.8 ± 4.2
LAK+SP	HT-29	11	1.2 ± 0.5	4.1 ± 0.9	9.3 ± 1.0	16.9 ± 3.1
LAK+SP	Fresh Ca	8	5.6 ± 1.2	12.6 ± 3.3	$38.3 \pm 6.3^*$	$59.4 \pm 9.5^*$
LAK	Fresh Ca	11	$0.0 \pm 0.0^*$	$0.1 \pm 0.1^*$	$0.1 \pm 0.1^*$	$0.5 \pm 0.5^*$

Substance P significantly increases LAK cell cytotoxicity against fresh colon cancer cells, but not against cultured cells.

RESUME

Les lymphocytes intestinaux sont influencés par les hormones intestinales. Nous avons postulé que la cytotoxicité des cellules LAK (lymphokine-activated-killer) de l'intestin pouvait être augmentée par la substance P (SP).

Nous avons isolé des LPMC (lamina propria mononuclear cells) et des cellules de cancer du colon provenant des spécimens opératoires par une méthode enzymatique et mécanique. Les cellules LAK furent induites en cultivant les LPMC avec 250 u/ml d'IL-2 (interleukin-2). Une concentration de 10^{-5} de substance P fut ajouté au médium de culture. Comme cibles nous avons utilisé des cellules du cancer du colon fraîches, HT-29 (une lignée de cellules cancéreuses humaines gardées en culture); et une lignée de contrôle. Après 4 jours d'incubation, nous avons mesuré la cytotoxicité à l'aide d'un ^{51}Cr release assay" de 4 heures.

Les cellules LAK démontrèrent une cytotoxicité modérée contre HT-29 et aucune envers les cellules cancéreuses fraîches. Les cellules LAK cultivées avec de la substance P ont démontré de nouveau une cytotoxicité modérée contre HT-29 mais cette fois la cytotoxicité envers les cellules fraîches était forte. Le pourcentage de cytotoxicité $\pm$ SEM à divers ratios de cellules fut:
(* indique $p < 0.05$ comparé à la ligne du dessus).

Cellules	Cible	N	5:1	10:1	25:1	50:1
LAK	HT-29	7	2.8 ± 0.5	11.6 ± 1.0	12.3 ± 2.3	17.8 ± 4.2
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LAK	Fresh Ca	11	$0.0 \pm 0.0^*$	$0.1 \pm 0.1^*$	$0.1 \pm 0.1^*$	$0.5 \pm 0.5^*$

Nous concluons que la substance P augmente de façon significative la cytotoxicité des cellules LAK contre les cellules de cancer du colon fraîches mais pas celles gardées en culture.

INTRODUCTION

Colon carcinoma is a devastating disease. In North American males, it is only preceded by lung cancer as a cause of cancer death; in North American females it is the third cause of cancer death after lung and breast cancers.¹ Colon cancer is responsible for 20% of all cancer deaths.¹ To this day, the only curative therapy consists of definitive surgery with removal of all malignant tissue including isolated metastatic disease.

Because it is such a prevalent and lethal disease, it is important to identify patients with pre-malignant lesions or early disease. Despite all the efforts being deployed to diagnose colorectal cancer early in the hope of curative surgery, the majority of cases are diagnosed once the disease has already become invasive. This may explain the failure to improve survival rates for colorectal cancer in the last thirty years.² In addition, 50% of patients having surgery with curative intent will have a recurrence within 5 years and most patients undergoing a second resection will die of their disease.²

Adjuvant therapy is administered in addition to surgical treatment to the primary colorectal cancer with the intent to improve outcome. It is normally given to patients who have disease extending beyond the margins of surgical resection. It can be given to both patients with advanced local disease and metastatic disease.⁶⁰ There are several forms of adjuvant therapy: the more conventional modalities include radiotherapy and chemotherapy; the more innovative and experimental tools consist of hormonal manipulation and immunotherapy.

A- COLORECTAL CANCER: CONVENTIONAL ADJUVANT THERAPY (Radiotherapy, Chemotherapy and hormone therapy)

Individual adjuvant treatment modalities are selected for precise indications and each has its limitations.

Radiation has been found to be an effective treatment in the local and regional control of cancers in the colon and rectum. It is relevant to both large bowel and rectal carcinomas as local recurrence is an important cause of morbidity and mortality in both these diseases.³ Cass evaluated 165 patients with locally recurrent cancer.⁴ Sixty-three had local recurrence alone, 15 had distant metastasis to the liver and 92% of the local recurrences developed in contiguous structure of the original tumor. Any form of treatment that is addressing local control is therefore very valuable. Radiation alone can be used to treat rectal cancer fixed to pelvic structures or for palliation for locally inoperable cancer.³ There is evidence that pre-operative radiation therapy may decrease the rate of local recurrence.^{2,3} Unfortunately, the dosage that can be administered is restricted by the many structures in the abdomen that have a limited tolerance to radiation, particularly the small bowel. Chemotherapy and radiotherapy combined seem to be more effective in reducing overall and local recurrence than radiation alone.³

Chemotherapy has been applied most often in the context of systemic therapy for metastatic disease or as adjuvant therapy after resection of a primary tumor.⁵ The standard chemotherapeutic agents available for the treatment of colorectal cancer are 5-FU and leucovorin.^{5,60} The cytotoxicity of 5-FU can be augmented by leucovorin.⁵ It allows to obtain a desired effect using a lower dose of 5-FU thus minimizing its toxicity, which is primarily myelosuppression.⁵

Historically, response rates in the treatment of disseminated disease have been less than 20%. Recently several studies have been conducted suggesting that more intensive treatment regimens yield higher response rates.⁵ Combination of methotrexate plus 5-FU and

cisplatin plus 5-FU have been tried but the results are not superior to 5-FU alone.⁵ A trial was done to assess the efficacy of 5-FU and levamisole. Results showed a clear benefit.⁶ This combination is effective in prolonging relapse-free and overall survival.^{6,7} Nonetheless, 40% of patients treated with this regimen will relapse.⁷

For patients with isolated hepatic metastases, intra-arterial hepatic infusion of 5-FU has shown a higher response rate and prolonged survival compared with systemic administration.^{2,5} However, toxicity remains a significant problem. Portal vein infusion has also been attempted and is much less effective than hepatic artery infusion.⁵

5-FU is a radiosensitizer explaining the better results when the two modalities are combined.^{3,5} As an adjuvant modality, chemotherapy with 5-FU or combination of several agents brings at best a modest increase in survival for colon cancer. Rectal cancer on the other hand, is much more clearly sensitive to adjuvant therapy and this latter form of treatment is clearly indicated for rectal disease.⁵ This discrepancy in response to treatment is best explained by the fact that colon and rectal cancers are two different diseases despite the fact that they are often discussed together. Colon cancer, because of the anatomy, presents usually little difficulty in achieving complete removal of the primary tumor. On the other hand, because of the confines of the pelvis, rectal cancer spreads to the lateral pelvic walls rapidly such that surgically resecting all local disease is more difficult. In this setting, adjuvant therapy can be very useful.

In summary, neither radiotherapy nor chemotherapy, alone or in combination, is currently successful at curing colorectal cancer. They cannot be depended upon when surgery is not possible or has failed; neither is successful in prolonging life significantly. While the effort to develop new chemotherapeutic agents and to optimize the doses and modes of delivery of radiation is still ongoing, new and different modalities are being actively explored.

Hormonal manipulation is a budding field which rests on in vitro and in vivo observations in/on experimental animals, that some hormones and growth factors promote tumor growth and proliferation.⁸ Clinical applications are used daily in the treatment of breast and prostatic cancer. Some breast cancers are dependant on ovarian hormones for growth.⁹ Women whose primary tumor is estrogen-receptor-positive respond to anti-estrogen therapy. The nonsteroidal antiestrogen tamoxifen is the endocrine treatment of choice for breast cancer.¹⁰ Trials have shown a disease-free and overall survival advantage at 5 years for patients with estrogen-receptor-positive primary tumors after 3 years of tamoxifen.¹¹ Similarly prostatic cancer is dependant on androgens for growth.¹² But in contrast to breast cancer, the correlation between androgen receptor status of the prostatic tumor and hormonal therapy is not well established.^{13,14} Anti-androgen therapy for prostatic cancer is nonetheless effective and several methods are available: surgical castration; medical castration with estrogens; LHRH agonists or ketoconazole; androgen-receptor antagonists such as flutamide.¹² Work is also being done in the field of colorectal cancer with substances like gastrin.⁸ Gastrin promotes the growth of some gastric and colonic carcinomas in vitro.¹⁵ Gastrin acts by binding to specific surface receptors which have been demonstrated on cancer cells from human colon.¹⁶ Hormonal manipulation through gastrin receptor antagonism as a viable therapeutic tool awaits further experimentation.⁸

B- IMMUNOTHERAPY IN COLORECTAL CANCER

1- BACKGROUND

Immunity plays a central role in defense against cancer. Immunotherapy is based on the theory that a tumor is allowed to proliferate because it has escaped immune surveillance. To be recognized and eliminated, a tumor cell must be considered "*non-self*" by the immune system. If the characteristics of the tumor change and become "*self*", it will no longer be challenged by the

immune system. Another possibility is that the tumor escapes immune surveillance because the immune system is suppressed. The site of suppression could be at the level of tumor recognition or in response to it. Immunotherapy is therefore based on the premise that our body's immune system can be stimulated or manipulated out of suppression to eradicate residual local disease after surgical resection or widespread metastatic disease. Interest in immunotherapy was renewed in the past decade because of major technologic advances in immunology and molecular biology that have facilitated the identification, isolation and large-scale production of substances that may enhance the immune response to tumor.¹⁷

Several basic differences distinguish and separate immunotherapy from the more conventional treatment options. Radiotherapy and chemotherapy kill tumor cells in a log mode i.e they always kill a fraction of the tumor mass. They can never eradicate the entire tumor burden even if it is small. This mechanism of action does not apply to immunotherapy as all tumor cells could theoretically be destroyed. Practically, the tumor burden would have to be small enough to allow immune effector cells to make contact with all tumor cells.¹⁸ There is heterogeneity of tumor cells in the same patient and tumor cells develop resistance against chemotherapeutic drugs. Immunotherapy has the potential to circumvent these issues.¹⁹

2- ACTIVE IMMUNOTHERAPY

The concept of active specific immunotherapy dates from the 1960's. The rationale for active specific immunotherapy is based on the premise that immunization with unique proteins on the tumor cell surface will elicit a host immune response that results in lethality for the tumor cells but spares normal tissue.²⁰ The major limitation rests with weakly immunogenic tumors: colorectal cancer is one of those.²¹ To circumvent this problem, adjuvants such as *Bacillus Calmette-Guerin* (BCG) or *Corynebacterium parvum* were

employed in the hope that general immune stimulation would concomitantly increase the host anti-tumor response.¹⁹ Clinical attempts were unsuccessful and have been largely abandoned.²² The current focus of active specific immunotherapy for colorectal cancer is to use potent allogeneic or generic vaccines to tumor-associated antigens (TAA) common to most colorectal tumors. The main challenge with this approach is to identify these common tumor-associated antigens and to prepare potent vaccines that recognize them. A phase I trial has shown a median survival of 57 months in 28 patients, including 9 with widespread metastatic disease.²³ Five of those 9 patients survived over 5 years.²³ These results are encouraging when compared with the overall 5-year survival of 1-2% reported for patients with metastatic colon cancer.²⁴

3- MONOCLONAL ANTIBODIES

Monoclonal antibodies (MoAbs) hold a great deal of promise in the treatment of cancer because of their high degree of specificity compared with chemotherapy. The most widely studied MoAb in colorectal cancer is the murine MoAb 17-1A, an immunoglobulin (Ig)G2a molecule that recognizes a TAA (CO17-1A) expressed by virtually all colorectal carcinoma cells. Unfortunately, objective response rates have generally been low (around 5%) with generally short duration of response and few complete responses.²³ Because of their high degree of specificity, MoAbs can be linked to potent toxins that either could not be employed systemically or that have potent short-range activity. Examples include conjugates with radiopharmaceuticals, chemotherapeutic agents, and immunotoxins.

4- ADOPTIVE IMMUNOTHERAPY

Adoptive immunotherapy is defined as the transfer to the tumor-bearing host of active immunologic reagents, such as cells, with anti-tumor reactivity.²¹ These reagents can mediate anti-tumor effects directly or indirectly.²¹ Because the body's defense

against malignant processes seem to rely primarily on the cell-mediated arm of the immune system, the agents transferred to the host have mainly consisted of T cells.¹⁷ The definite advantages of adoptive immunotherapy are that the immunosuppression generally associated with the tumor bearing state is not a problem; since the transferred reagents are immunologically specific, this treatment should have a high degree of specificity and consequently a low morbidity; no immunosuppression should be caused by this treatment.¹⁷ There are two limitations to adoptive immunotherapy: first a large number of cells are required for transfer; and second, there is great difficulty in generating specific immune cells against all tumors.¹⁷

The cornerstone of adoptive immunotherapy has been the lymphokine-activated-killer cell (LAK cell). It is one of the three type of cytotoxic T cells. The other two are the cytotoxic T cell and the NK (natural killer) cell. The former is Class I MHC restricted.

LAK cells are T cells that become activated in the presence of interleukin-2 (IL-2). LAK cells are not MHC restricted nor restricted to a specific tumor type.¹⁷ IL-2 is a 15 000 daltons glycoprotein secreted by activated T cells. It was initially termed "T-cell growth factor" because of its ability to sustain the proliferation of activated T cells in long term cultures. Recently its gene has been cloned and large quantities of the recombinant product are now available. IL-2 induces or enhances the secretion of other cytokines including interferon alpha, hematopoietic colony-stimulating factors, interleukin-1, tumor necrosis factor (TNF), and B-cell growth factor.¹⁷

Rosenberg et al. at NIH are responsible for much of the pioneer work with LAK cells and IL-2. Numerous studies have been done in animals, mainly in the mouse model. Several murine tumor lines have been used, including a series of variably immunogenic methylcholanthrene (MCA)-induced sarcomas. Inoculation of a few sarcoma cells into a mouse's tail vein results in pulmonary, hepatic, and peritoneal metastatic lesions at 3 days, which are grossly visible at 10 days.¹⁷ LAK cells infused alone were largely ineffective

against 3-day and 10-day metastatic lesions, but the co-administration of LAK cells and IL-2 resulted in a dramatic reduction in tumor nodules in the lungs and livers of these animals.²⁵⁻²⁷ Several clinical trials have also been conducted in humans. They have shown that the administration of LAK cells alone was not effective in humans.¹⁷ Other trials were done with IL-2 alone: in the first trial with high-dose IL-2,²⁸ 10 patients were treated, and 3 of 6 patients with melanoma had a partial response, defined as a 50% or greater reduction in tumor burden. This result led to trials of high-dose IL-2 in combination with LAK cells. Several nonrandomized and randomized trials of IL-2 alone or in combination with LAK cells were done at the National Cancer Institute (Table 1).²⁹ The overall response rates, defined as complete and partial responses, were 21% and 16%, respectively.^{17,30} Most responses occurred in patients with renal cell carcinoma and melanoma.^{17,29,30,31,32,33} IL-2 alone was not as effective as the combination of IL-2 and LAK cells.^{17,29,30,33}

Colon cancer exhibits a minimal response to these highly competent immune cells.^{29,34,35}

TABLE 1. Results of Nonrandomized and Randomized trials of Interleukin-2 (IL-2) Alone or in Combination with Lymphokine-Activated-Killer (LAK) Cells in Patients with various types of Cancer²⁹

Type of cancer	Type of therapy	No. of patients	Overall response		Response	
			No	%	Complete	Partial

			Nonrandomized trial			
Renal carcinoma	IL-2/LAK	54	17	31	7	10
	IL-2	38	7	18	4	3
Melanoma	IL-2/LAK	34	6	18	3	3
	IL-2	23	6	26	0	6
Colorectal cancer	IL-2/LAK	27	3	11	1	2
	IL-2	10	0		0	0
Non-Hodgkin's lymphoma	IL-2/LAK	4	3	75	1	2
	IL-2	3	0		0	0
			Randomized trial			
Renal carcinoma	IL-2/LAK	34	10	29	6	4
	IL-2	31	5	16	3	2
Melanoma	IL-2/LAK	16	2	12	2	0
	IL-2	12	4	33	0	4
Colorectal cancer	IL-2/LAK	5	0		0	0
	IL-2	7	0		0	0
Non-Hodgkin's lymphoma	IL-2/LAK	2	1	50	0	1
	IL-2	1	0		0	0

C- THE PROBLEMS OF COLORECTAL CANCER

Why is colon cancer resistant to immunotherapy with LAK cells? Three hypothesis have been formulated. Firstly, this could be due to poor interaction between LAK cells and their colonic targets. Most clinical studies utilized lymphocytes isolated from the circulation to generate LAK cells. Trudel et al.³⁶ postulated that lymphocytes from the intestinal lamina propria could represent a more relevant source of immune cells. Colon cancer cells turned out to be as resistant to lysis by lymphocytes from an intestinal source as they had been to lymphocytes isolated from the circulation.³⁶ Secondly, colon cancer could be resistant to LAK cell therapy because of intrinsic tumor resistance. Unfortunately, tumor dynamics are not well enough understood at the present time to allow their modification. Thirdly, the lack of response could be attributable to insufficient LAK cell cytotoxicity. This can be modulated and it constitutes the basis of this thesis.

D- HORMONAL MODULATION

Gut-associated lymphoid cells and circulating immune cells are modified by several peptides originally described as neurotransmitters or gut hormones.^{37,38} This opens the possibility of using hormonal manipulation to improve LAK cell cytotoxicity against colon cancer. Although the exact mechanism through which neuropeptides and gut hormones modify the immune function is not entirely understood, several facts have been documented. Somatostatin (SMS) inhibits lymphocyte proliferation and stimulates antibody-dependant cellular cytotoxicity in-vitro.^{39,40} Substance P stimulates human T lymphocyte proliferation in-vitro.⁴¹ β -endorphins enhance the proliferative response of T lymphocytes and stimulate chemotaxis in monocytes and neutrophils.^{42,43} The presence of high-affinity hormonal receptors on lymphocytes suggests the possibility that modulation of gut mucosal immunity can be achieved via a direct effect on lymphocytes. A subset of lymphocytes has been shown to possess high-affinity receptors for

substance P; SMS receptors have been identified on peripheral blood lymphocytes; and β -endorphins are known to exert their action through surface receptors.^{44,45,46,47} Although further investigations are required to clarify the nature of hormone-immune cell interactions, their existence is no longer questioned.

E- PURPOSE

The purpose of our project was to attempt to modulate gut mucosal immunity with various gut hormones. We used lymphocytes isolated from the intestinal lamina propria and attempted to augment their cytotoxicity. There are over thirty hormones which have effects on the gut. We have chosen to study substance P, because its effect on non-intestinal immune cells is well documented.^{41,42,43} Gut lymphocytes carry surface receptors for this hormone.⁴⁵ The mere presence of surface receptors for these hormones on gut lymphocytes strongly suggests that they do in fact play a role. This project will help shed some light on the effects of gut hormones on gut mucosal immunity. If hormonal modulation of gut mucosal immunity is a viable hypothesis, this could open a new exciting avenue for adoptive immunotherapy in the treatment of colorectal cancer.

MATERIALS AND METHODS

A- PATIENT POPULATION

Freshly resected intestinal tissue was obtained from operative specimens of patients undergoing bowel resection for cancer or benign colonic conditions at the Montreal General Hospital, the Royal Victoria Hospital and St-Mary's Hospital. An agreement was made with the respective Departments of Pathology to ensure that the needs for tissue did not interfere with staging of the tumor in any way.

Lamina propria mononuclear cells (LPMC) were isolated from 19 patients. Ten of them had colorectal cancer: 5 had sigmoid lesions, 3 had rectal lesions and 2 had cecal tumors. Three patients had surgery for excision of a villous tumor or multiple polyps, 3 had diverticular disease, 1 had Crohn's disease, 1 had ulcerative colitis and 1 had intractable lower gastrointestinal hemorrhage. The patients who were operated on for cancer also had part of the resected specimen utilized for isolation of fresh cancer cells.

B- ISOLATION AND PURIFICATION OF GUT MUCOSAL LYMPHOCYTES (GML)

Gut mucosal lymphocytes (GML) were isolated using Fiocchi's modification⁴⁸ of the original technique described by Bull and Bookman.⁴⁹ The mucosa was dissected from the rest of the bowel wall and cut in fragments measuring approximately 1 cm². The fragments were freed of mucus and epithelial cells in sequential steps, using 75 mg of dithiothreitol (DTT; Sigma Chemical Co., St-Louis MO) in 50 ml of calcium and magnesium free Hank's balanced salt solution (CMF-HBSS; GIBCO) for thirty minutes and 37,5 mg of ethylenediaminetetraacetic acid (EDTA; Sigma) in 90 ml of CMF-HBSS for two periods of ninety minutes. Overnight digestion in calcium and magnesium free Hank's balanced salt solution (CMF-HBSS) containing 0.01% collagenase and 0.01% deoxyribonuclease (both from Worthington Diagnostic system, Inc., Freehold NJ) was

performed. The resulting crude cell suspension was filtered and purified over a Ficoll-Hypaque gradient (Pharmacia). The isolated cells were almost exclusively LPMC (90-95% purity, verified by microscopic examination).

C- INDUCTION OF LAK CELL ACTIVITY FROM GML

Cells designated for induction of LAK cells were put in RPMI-1640 culture medium (GIBCO) with 10% human AB serum (GIBCO), 1.5% Hepes buffer (GIBCO), 1% gentamicin, 2.5% penicillin-streptomycin-fungizone (PSF, GIBCO). Recombinant human IL-2 (Cetus Corporation, Belmont CA) was added at a concentration of 250 units/ml. The cells were cultured at a concentration of $0.5 \times 10^6/\text{ml}$ in upright tissue culture flasks at 37°C in a 5% CO_2 -95% humidified air atmosphere for a period of 4 days. In order to establish that the cells resulting from this culture were indeed LAK cells, we first cultured the intestinal lymphocytes without IL-2. When an assay was done against K562 and Daudi, K562 was sensitive to these lymphocytes whereas Daudi was resistant. Then the gut lymphocytes were cultured with IL-2 as described above. Repeat assays indicated that these cells could kill both K562 and Daudi establishing that they were indeed LAK cells.

D- HORMONAL ENHANCEMENT OF GML

Substance P (Peninsula Labs, San Carlos, CA) was added to the tissue flasks simultaneously with induction of LAK activity. A concentration of 10^{-5}M was selected after going through the following determinations: in the gastrointestinal tract, concentrations as low as 10^{-10}M exert a physiologic effect.⁴⁵ This concentration was therefore used as the starting point and was increased until a response was obtained. 10^{-5}M therefore represents the threshold dose in the dose-response curve; it was selected over a higher dose because it is already pharmacological. Other in vitro studies using Substance P as an immunomodulator used concentrations ranging between 10^{-10}M and 10^{-6}M .¹¹

E- ISOLATION OF FRESH COLORECTAL CANCER CELLS

The procedure is a combination of the methods of Brattain et al.⁵⁰ and Keder et al.⁵¹ Non-necrotic malignant tissue was washed in CMF-IIBSS and minced in 3-5 mm fragments. The fragments were placed in an enzymatic solution containing hyaluronidase (0.5 mg/ml, Sigma), collagenase and DNAase (2mg/ml and 0.6mg/ml respectively, Worthington). This digestion process was repeated approximately 5 times for successive periods of 45 min. The cell suspension from the first digestion period was discarded to reduce/eliminate contamination with red blood cells, dead cells and debris. The cells were then collected by centrifugation and further purified over a 20% Percoll gradient (Pharmacia). The cells isolated were usually 85-90% viable (verified by microscopy) and were either used immediately or frozen in liquid nitrogen.

F- TARGET CELL LINES

Several target cell lines were used for cytotoxicity assays. Daudi is a lymphoid cell line used as a LAK sensitive internal control. The cultured human colorectal cell line HT-29 was maintained in duplicate in a solution of RPMI-1640 supplemented with 10% fetal calf serum (FCS; GIBCO) and 1% PSF and split every third day. It is known to be slightly LAK sensitive and was used as one of the experimental targets.³⁶ Fresh colon cancer cells isolated from human surgical specimens were used as the other experimental target.

G- RADIOACTIVE LABELING OF TARGET CELL LINES

Target cells were labeled by incubation with Chromium 51 (⁵¹Cr). Three to five X 10⁶ cells were harvested and pelleted by centrifugation (1700 rpm X 5 min.). All but 2 ml of supernatant was aspirated and 200 µCi of sodium chromate (⁵¹Cr; ICN Chemicals) was added.

The cells were incubated at 37° C for 90 min. and agitated periodically. In the case of adherent targets (HT-29), the cells were aliquoted in flat bottom microtiter plates at a concentration of 10^4 cells/0.2 ml/well and incubated overnight in a 5%CO₂-95% humidified air atmosphere to allow adherence. The next day, each well was washed 3 times immediately prior to the cytotoxicity assay. For suspended targets (Daudi, fresh cancer cells), the cells were washed 3 times and aliquoted in a round bottom microtiter plate at a concentration of 10^4 cells/0.2 ml/well at the time of the assay.

H- MICROCYTOTOXICITY ASSAY FOR LAK CELL ACTIVITY

Effector LAK cells, cultured with or without substance P, were added in triplicate to the wells containing target cells. For flat bottom plates, a volume of 0.2 ml/well was used and for round bottom plates, a volume of 0.1 ml/well was used.

Various effector to target (E:T) ratios (5:1, 10:1, 25:1, 50:1) were obtained by serial dilution of the effector cells added to a fixed number (10^4) of ⁵¹Cr-labeled target cells/well. Spontaneous and maximal release were determined respectively by addition of culture medium and 1% sodium dodecyl sulfate (SDS, Sigma). Microtiter plates were centrifuged at 500 rpm for 3 min and incubated at 37°C in a 5%CO₂-95% humidified air atmosphere for 4 hours. Following incubation, plates were spun again at 1000 rpm for 5 min, 0.1 ml of supernatant was aspirated from each well, and radioactivity was measured as counts per minute (cpm) in a Gamma counter. Cytotoxicity was expressed as % specific ⁵¹Cr release using the following formula:

$$\% \text{ specific release} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

All statistical analyses were done using a two-tailed, unpaired student's t-test and differences were considered significant if $p < 0.05$.

RESULTS

A) LAK COMPARED TO LAK + SP AGAINST HT-29

The cytotoxicity of LAK cells alone was compared to the cytotoxicity of LAK cells cultured with Substance P (SP) against the colon cancer cell line in culture HT-29. For the control group (LAK cells alone), we did 7 assays. The mean age of the patients from whom the lymphocytes were obtained was 62 years. There were 3 males and 4 females. Four patients had colon cancer, 1 had diverticular disease, 1 had unexplained lower gastrointestinal bleeding and 1 had multiple colonic polyps. In the experimental group (LAK cells + SP), 11 assays were done. Patients' mean age was 60 years, there were 4 males and 7 females. The indication for surgery was: colon cancer in 6 patients, diverticular disease in 2, multiple polyps in 2 and Crohn's disease in 1. Trudel et al.³⁶ have characterized the effect of LAK cells alone against HT-29. We repeated these experiments and obtained similar results (Fig. 1). When we repeated the experiments with LAK cells cultured in the presence of substance P ($10^{-5}M$), we obtained the following results: the cultured colon carcinoma cell line HT-29 showed the same susceptibility to lysis by LAK cells cultured with substance P as it had to LAK cells alone (fig. 1). There was no statistical difference in the degree of cytotoxicity against HT-29 of LAK cells alone compared to LAK cells cultured with SP ($p=0.15$) (Fig. 2). There was also no significant difference across the 2 groups between patients' age, sex, indication for bowel resection, Duke's stage of the disease and histological grade of the tumor.

FIGURE 1

FIGURE 2

B) LAK COMPARED TO LAK + SP AGAINST FRESH COLON CANCER

The cytotoxicity of LAK cells alone was compared to that of LAK cells cultured with Substance P (SP) against fresh colon cancer cells. In the control group (LAK cells alone), 8 assays were done. There were 3 males, 5 females and their mean age was 63 years. Surgical indications were: 6 carcinomas of the colon, 1 Crohn's disease and 1 diverticular disease. In the experimental group (LAK cells + SP), 11 assays were done. Five patients were males 6 females and their mean age was 59 years. Seven underwent surgery for colorectal cancer, 2 for diverticular disease, 1 for multiple colonic polyps and 1 for Crohn's disease. As Trudel et al.¹⁸ had shown, we found fresh colon cancer cells to be completely resistant to LAK cells alone (fig. 3). In contrast, fresh colon cancer cells were lysed by LAK cells cultured with substance P in a dose-responsive fashion (fig. 3). At each effector to target (E:T) ratio studied, there was a statistically significant difference in the cytotoxicity of LAK cells cultured with SP compared to LAK cells alone ($p < 0.01$) (Fig.4). The results obtained are independent of the age and sex of patients and of the clinical indication for bowel resection. These results are also independent of the Duke's stage of the disease, of the histological classification of the malignancy and of the autologous or allogenic nature of the effector cells. To control for the possibility that substance P itself could be the lethal agent, we performed 3 assays where the effector was substance P alone with culture medium. When tested against fresh colon cancer cells in the same type of cytotoxicity assay as described above, there was no lysis of the fresh cancer cells by substance P.

FIGURE 3

FIGURE 4

C) COMPARISON OF ACTIVITY OF LAK + SP AGAINST HT-29
AND FRESH COLON CANCER CELLS

The difference in the degree of cytotoxicity of LAK cells cultured with Substance P (SP) against fresh and cultured targets was compared. LAK cells cultured with SP exhibited a stronger degree of cytotoxicity toward fresh colon cancer cells than they did against the cultured colon cancer cells HT-29. At higher E:T ratios (25:1, 50:1), the difference was statistically significant ($p < 0.01$) (Figs. 5 & 6).

FIGURE 5

FIGURE 6

DISCUSSION

The results of this study suggest that the gut mucosal immune system can be modulated by substance P. The net effect of this modulation results in an increase in gut-derived LAK cell cytotoxicity against fresh colon cancer cells, but not against the cultured human colon cancer cell line HT-29.

The mechanism for modulation is uncertain at this time. Previous reports have demonstrated specific stimulation of human peripheral blood T-lymphocytes by Substance P.^{42,43,45} Other reports have demonstrated a stimulatory effect of SP on human thymus⁵², and on human B lymphocytes⁵³. Animal studies have also reported a modulatory effect of SP on the immune system: Moore et al. have worked on lymph nodes of sheep^{54,55} and demonstrated an increase in CD4 cells mediated by SP. Croitoru et al. have done experiments with murine intestinal lymphocytes. They have shown an increase in the natural killer activity modulated by SP⁵⁶.

In addition to these several studies that demonstrated a stimulatory effect of SP on a number of lymphocyte subsets, the presence of high-affinity receptors for SP on lymphocytes has been suggested.^{41,45,57} This body of information led to the hypothesis that SP exerts its effect on lymphocytes via a direct hormone-receptor interaction. This theory is supported by the fact that an antagonist of SP; the peptide [D-Pro²,D-Phe⁷,D-Trp⁹]-SP inhibits the proliferative effect of SP on human peripheral blood lymphocytes by a competitive mechanism.¹⁴ This antagonist was first characterized as such when it was found to inhibit the SP-induced contraction of guinea pig ileal smooth muscle.^{58,59}

In summary, so far, the literature is capable of supporting the theory of a receptor-mediated stimulatory effect of Substance P on human peripheral blood lymphocytes. However we have done a series of experiments using intestinal lymphocytes, not peripheral blood lymphocytes. To determine if the modulatory effect of SP on this particular subset of immune cells is also receptor-mediated, we are currently conducting a series of experiments whereby a group of

LAK cells are cultured with substance P alone and a second group of LAK cells is cultured with substance P and its antagonist. If the results of this experiment show competitive inhibition, we will have demonstrated that the effect of substance P on intestinal lymphocytes is also receptor-mediated.

Our results showed increased LAK cell activity by hormonal manipulation with SP against a fresh but not against a cultured target. Why do fresh cancer cells and cultured cells respond differently to the same effector cells? To our knowledge there has been no work done on the effect of neuropeptides such as substance P on colorectal cancer. We do not know if there is a specific SP receptor on colon cancer cells. Should there be a specific SP receptor on human colon cancer, the presence of SP in the culture medium could improve the stereotaxic interaction between the effector and its target. Substance P receptors might be down-regulated on cells kept in culture for extended periods, which would prevent the improved interaction to take place. We are presently culturing the HT-29 target cell line with Substance P in order to perhaps up-regulate SP receptors in these cells. We will then repeat the microcytotoxicity assays with LAK cells + SP against HT-29 + SP. This should help to determine the mechanism of action of SP and its interactions with target cells.

In summary we have demonstrated an enhancing effect of Substance P on gut-derived cytotoxicity against fresh colon cancer cells. From these first experiments we conclude that the hypothesis of hormonally manipulating the gut mucosal immune system is viable. Previous work has shown a receptor-mediated effect of SP on peripheral blood lymphocytes but not on intestinal lymphocytes. We need to do additional work to determine if the effect of SP on the LPMC is also receptor-mediated by using SP's antagonist. We need, in light of the results we obtained, to do a series of assays using peripheral blood lymphocytes of patients with colon cancer. Should we observe similar findings, blood lymphocytes represent a more accessible source of cells when clinical application is

considered. We also need to characterize the way LPMC enhanced by SP interact with their target in order to explain the different results observed in fresh and cultured colon cancer cells.

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