Characterization of Cell-Mediated Immunity in Long-Term Survivors of Gastric or Colorectal Cancer

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The prognosis of patients with neoplastic disease seems to be influenced by several factors of tumor and host origin [*1*]. For example, gastric and colorectal adenocarcinomas vary in prognosis, depending on factors such as the degree of penetration of the tumor into the stomach or bowel wall, the number and location of lymph nodes involved, and the degree of differentation of the lesion [*2,3*]. On the other hand, the relationship between host immune competence and tumor growth has also been the subject of extensive studies [*4,5*]. Although, depression of cellular immune reactivity is observed mainly in patients with disseminated gastric or colorectal carcinoma, the role played by the immune system regarding the length of survival of these patients is still a matter of controversy [*6–8*]. We have investigated cellular immune mechanisms in long-term survivors who have been considered clinically cured of advanced gastric or colorectal carcinomas, including the influence of autologous patient serum on in vitro recognitive and lymphoproliferative responses.

Material and Methods

Twelve survivors who had gastric and colorectal cancer were studied. TNM classification [*9*] at time of surgical treatment, stage, and length of survival are listed in Table I. All patients were treated by extensive surgical excision and 1 year of postoperative chemotherapy at least 5 years before this study was undertaken. Patients 9 and 12 had multiple carcinomas. Patient 2 consented to surgical treatment 12 months after the diagnosis was made. Patients studied had no evidence of neoplastic disease (tumor recurrence or metastasis) as determined by clinical symptoms, physical examination, blood tests, roentgenograms (chest and gastrointestinal tract), gastrointestinal endoscopy, scintillation liver scan, and serum levels of carcinoembryonic and α-fetoprotein antigens documented by radioimmunoassay.

Peripheral blood lymphocytes were prepared from 30 ml of fresh heparinized blood (10 units of free preservative heparin per milliliter of blood). The blood was diluted with saline solution (1:1) and was layered over Ficoll-Hypaque (Pharmacia, Upssala, Sweden); the interface was removed, washed twice, and then resuspended in RPMI 1640 medium (Microbiological Associates, Bethesda, MD) supplemented with 25 mM, Hepes buffer, a penicillin and streptomycin mixture (100 IU/ml), 2 percent of 2 mM L-glutamine, and 2 percent of heat-inactivated, pooled human serum. Two healthy control subjects from either the blood bank or our laboratory were tested simultaneously with each patient. Before the assays were performed, the peripheral blood lymphocytes were incubated overnight (precultured cells).

Ten milliliters of blood was drawn from each patient. The blood was set at room temperature for 1 hour, the clot was removed, and after centrifugation, the serum was separated and heat inactivated for use in the in vitro test. Four fresh samples were frozen at −70°C to determine the presence of immune complexes.

The counting of T-lymphocytes was performed by E-rosette formation as described, using sheep red blood cells [*10*]. The percentage of rosettes stained with cresyl blue was determined directly.

To obtain lymphocyte proliferation in vitro, mitogen stimulation was induced by placing 2×10^5 cells into each well of flat-bottom Microtest II® plates (Falcon Plastics, Inc., Oxnard, CA) and adding a predetermined optimal concentration of phytohemagglutinin-M (5 μg/ml). (Calbiochem, San Diego, CA). Each experiment was tested in triplicate in the presence of pooled, heat-inactivated normal human serum or patient serum in a total volume of 200 μl. The cultures were incubated in a humidified atmosphere at 37°C with a 5 percent carbon dioxide air mixture

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TABLE I Characteristics of the Selected Patients

Patient No.	Age (yr) & Sex	TNM Classification*	Stage	Survival (yr)
		Gastric Adenocarcinoma		
1	58, F	$T_4N_2M_0R_0G_3$	IV	7.5
2	63, M	$T_3N_1M_0R_0G_2$	III	5
3	75, F	$T_3N_2M_0R_0G_2$	III	5.5
4	61, M	$T_3N_0M_0R_0G_1$	II	5
5	63, M	$T_3N_0M_0R_0G_x$	II	7
6	65, F	$T_2N_0M_0R_0G_1$	II	10
7	67, M	$T_4N_1M_0R_0G_1$	IV	5
		Colorectal Adenocarcinoma		
8	46, F	$T_5N_1M_0R_0G_2$	III	7
9†	52, F	$T_2N_1M_0R_0G_1$	III	5
10	70, M	$T_2N_1M_0R_0G_1$	III	5
11	54, F	$T_2N_1M_0R_0G_1$	III	8.5
12‡	75, M	$T_3N_xM_0R_0G_2$	II	5

* The histopathologic tumor grade (G_x, G_1, G_2, or G_3) and postsurgical treatment residual tumor (R) have been added to the otherwise standard TNM clinical staging system [9].

† Breast cancer, surgical treatment; survival was 7.5 years.

‡ Laryngeal cancer, thyroid cancer; surgical treatment; survival was 4 years and 7 years for the larynx and thyroid cancer, respectively.

for 72 hours. Eighteen hours before harvesting, the cultures in each well were pulsed with 1 μCi per well of radioactive thymidine with a specific activity of 2 Ci/mM (New England Nuclear, Boston, MA). The spontaneous incorporation of thymidine by lymphocytes was determined by culturing cells in the absence of mitogen.

To perform a mixed lymphocyte culture, 1×10^5 lymphocytes from the responding cell population were mixed with an equal amount of mitomycin (25 μg/ml) for 30 minutes (Sigma, St. Louis, MO), with treated lymphocytes being used as the stimulating population. Mixed lymphocyte cultures were performed using two control subjects and one patient. Each set of cells was used both as a stimulator and a responder with all cell preparations. Each experiment was carried out adding normal serum or patient serum in round-bottom Microtest II plates in triplicate in a final volume of 150 μl. The plates were incubated for 120 hours at 37°C in a humidified atmosphere of 5 percent carbon dioxide air mixture. Three experiments (one patient and two control subjects each) were set up comparing the mixed lymphocyte culture response of unseparated lymphocytes and the reactivity of enriched T- and non-T-lymphocyte populations obtained by rosette formation of human T-lymphocytes with sheep erythrocytes. The responding T-lymphocytes were mixed with mitomycin C treated allogeneic non-T-lymphocytes. Culture conditions were similar to those of the aforementioned mixed lymphocyte culture. Eighteen hours before harvesting, 1 μCi of radioactive thymidine was added to each well.

The cultures were harvested onto fiberglass filters with an automated multisample harvesting device (Mash II®, Microbiological Associates, Bethesda, MD), and the filters were counted in a liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, IL).

The antibody-dependent cellular cytotoxic activity of the peripheral blood lymphocytes from patients with colorectal adenocarcinoma was determined using RhD^+ erythrocytes as target cells, coated with specific immunoglobulin G anti-D (Ortho Diagnostic Inc., Raritan, NJ), as previously described [11]. Lymphocyte proliferation in mitogen cultures and in mixed lymphocyte cultures was expressed as a relative proliferation index, defined as the ratio between the net counts per minute of each patient and the mean net counts per minute of the normal subjects assayed simultaneously [12].

Results

All of the patients showed an insignificant decrease in the T-lymphocyte percentage (mean 58 percent) when compared with the control subjects assayed simultaneously (mean 64 percent). Both mitogen and allogeneic responses in vitro from the two groups of patients were compared with those of the concurrent control subjects set up simultaneously with each patient. The relative proliferation index values obtained in either patients or control subjects, were compared with values established in our laboratory for a lymphoblastic transformation test to phytohemagglutinin (88 control subjects, relative proliferation index ≤ 0.65) and for mixed lymphocyte culture (57 control subjects, relative proliferation index ≤ 0.66). As can be noted, proliferative responses to phytohemagglutinin were mostly preserved in the gastric adenocarcinoma group, whereas proliferative reactivity to alloantigens (mixed lymphocyte culture) was preserved in the majority of patients from both groups (Tables II and III). Both groups also remained normal in the assays using T-lymphocyte and non-T-lymphocyte enriched populations (Table IV).

All sera tested modified the patients in vitro responses to phytohemagglutinin or alloantigens (Table III). Inhibition or enhancing effects were

TABLE II Lymphocyte Proliferation In Vitro*

	No.	LTT	MLC
Patients	12	1 ± 0.3	0.82 ± 0.12
Gastric adenocarcinoma	7	0.97 ± 0.25	0.78 ± 0.14
Colorectal adenocarcinoma	5	1 ± 0.75	0.88 ± 0.23
Control subjects	24	0.98 ± 0.08	0.98 ± 0.06

* Lymphoblastic transformation test (LTT) results and mixed lymphocyte culture (MLC) are expressed by the relative proliferation index (mean ± standard error of the mean). Using the unpaired t test, no significant difference was determined among the groups.

observed. Furthermore, three gastric adenocarcinoma serum samples (samples 1, 2, and 4) and one rectal adenocarcinoma serum sample (sample 9) were able to induce a bimodal effect that either inhibited or facilitated the responses on the lymphoproliferative tests. In addition, serum samples 3 and 7 (gastric adenocarcinoma group) and serum sample 8 (rectal adenocarcinoma group) changed a normal in vitro response to subnormal.

Cytotoxic capacity was explored in the colorectal adenocarcinoma group. A normal or low capacity was found even in those survivors with multiple malignant tumors (Table V).

Comments

Assessment of cell-mediated immunity mainly through in vitro studies has shown that patients bearing gastric or colorectal carcinoma exhibit alterations in proliferative or effector responses [*8,11,13*]. In our laboratory, Blanca et al [*11*] reported preserved allogeneic reactivity in nonadvanced gastric carcinoma, whereas depressed allogeneic reactivity in advanced but resectable lesions was restored postoperatively. In colorectal cancer, several studies [*6–8,13*] have demonstrated that altered cell-mediated immunity reactivity was more prevalent in rectal cancer [*14*] and most often was correlated with the stage of the disease. Recently, Wanebo et al [*15*] reported a positive correlation between normal lymphocyte counts, proliferative response to mitogens, and longer survival in patients with distant colorectal cancer metastasis. It is still uncertain whether the altered cell-mediated immunity reactivity visualized in patients with neoplastic disease may appear before or after the tumor.

We have investigated the cell-mediated immunity competence in a group of long-term survivors of gastric or colorectal cancer. The T-lymphocyte percentage (E-rosettes) did not differ significantly from the normal range. Only one 75 year old patient who had three primary tumors, showed a T-lymphocyte count of 34 percent, which, in part, may have been due to a reduction in peripheral T-lymphocytes observed during senescence [*16,17*]. Proliferative responses were intact in 75 percent of the patients (in the presence of normal human serum), particularly the reactivity to alloantigens. Moreover, experiments performed with previous separation of peripheral mononuclear cells in enriched T-lymphocyte and non-T-lymphocyte fractions, showed a similar pattern of normal T-lymphocyte response to alloantigen stimulation. These results tend to indicate that the recognitive and lymphoproliferative phases of the immune response to alloantigen stimulation seem to be well preserved in long-term survivors of gastric and colorectal cancer.

Several studies have shown that in vitro cell-mediated immunity reactivity in patients with tumors may be influenced by autologous serum factors [*18*]. Most of these investigations have focused on the significant inhibitory effect of autologous serum on lymphoproliferative responses or leukocyte migration activity [*11,18*]. Our laboratory has also identified enhancing properties of autologous serum factors in

TABLE III Influence of Autologous Serum (AS) on Lymphocyte Proliferation In Vitro*

Patient No.	LTT		MLC		Enhancement (%)	Inhibition† (%)
	NHS	AS	NHS	AS		
1	0.77	0.24	0.51	0.78	35	69
2	0.93	0.65	0.87	1.3	33	70
3	1.7	0.16	0.97	0.5	...	70
4	0.57	1.1	0.44	0.35	93	20
5	0.61	3.3	0.66	1.5	284	...
6	1.9	0.72	1.4	1.2	...	38
7	0.33	0.06	0.66	0.44	...	58
8	0.31	0.03	1.2	0.04	...	79
9	0.55	0.74	0.72	0.18	35	75
10	3.8	0.82	1.6	0.89	...	61
11	0.41	0.69	0.39	1.2	69	...
12	0.15	0.1	0.5	0.24	...	43

No significant difference was determined by the paired t test.
† In Patients 1, 2, 4, and 9 there was a bimodal effect.
LTT = lymphoblastic transformation test results; MLC = mixed lymphocyte culture; NHS = normal human serum.

TABLE IV Mixed Lymphocyte Culture With Enriched T-Lymphocyte and Non-T-Lymphocyte Fractions*

	Peripheral Blood Lymphocytes	T- and Non-T - Lymphocyte Fraction†
Patients		
1	0.87	1
2	1.2	0.98
3	1.6	3
Control subjects‡		
1	0.78	0.94
2	1.2	1.4
3	0.69	3.2
4	1.3	5
5	1.3	0.95
6	0.98	1.4

* Mixed lymphocyte cultures expressed by the relative proliferation index (assays in normal human serum).
† Enriched populations by the E-rosette technique.
‡ Determinations made simultaneously with those of the patients.

TABLE V Antibody-Dependent Cellular Cytotoxic Activity in the Colorectal Adenocarcinoma Group*

Patient No.	K Values†
8	$>11 \times 10^5$
9	$>11 \times 10^5$
10	4×10^5
11	$>11 \times 10^5$
12	3×10^5

* The normal values for ADCC activity were established from 52 control subjects (0.9 to 11×10^5 cells).
† K values are represented by the number of effector cells required to mediate 50 percent of lysis.

gastric and lung cancer [*11,19*]. In our patients, each serum tested was able to modify its autologous in vitro cell response (both to phytohemagglutinin and alloantigens). The serum effect was variable, the main influence was bimodal, inducing either suppressive or enhancing effects. However, significant inhibition to subnormal relative proliferation index values was noted in only three patients. Two patients enhanced a significantly low proliferative response to within the normal range. These findings suggest perhaps, that the nature of autologous serum factors in long-term survivors of gastric and colorectal cancer may be different from those present in patients with tumors, which have frequently showed a strong inhibitory influence [*11,19*]. Within this context, circulating immune complexes have been implicated as one of the sources of blocking serum factors in malignancies [*20*]. Furthermore, in gastric or colorectal cancer, the presence of circulating immune complexes seems to be associated with advanced stages with or without metastatic disease, elevated levels of carcinoembryonic antigens and hyporesponsiveness to phytohemagglutinin or alloantigens [*11,21*]. In our patients, sera samples 4 and 9 showed a bimodal effect on the in vitro tests; serum sample 5 induced the highest percentage of enhancement, whereas serum sample 8 abrogated both proliferative responses to both phytohemagglutinin and alloantigens. All these samples were tested for circulating immune complexes showing levels similar to those of normal individuals as measured by the Raji cell or by Clq binding activity assays as previously determined in our laboratory [*22*]. Furthermore, none of the serum had an abnormal amount of carcinoembryonic antigen.

Increased attention has been given to the study of effector cell mechanisms in different types of tumors [*23,24*]. In relation to gastrointestinal cancer, the mechanism of cellular cytotoxicity against an antibody sensitized target has received particular attention. Hahn et al [*25*] reported that sera from their patients with colon cancer were active in killing cultured colon cancer cells by either complement-dependent cytotoxicity or antibody-dependent cell cytotoxicity. We have utilized the system of cellular cytotoxicity against human RhD^+ red blood cells previously sensitized with immunoglobulin G anti-D. Furthermore, like other workers [*26*], we believe that the nature of the effector cell in this antibody-dependent cell-mediated cytotoxicity system is the monocyte. Hersh et al [*26*] reported a high antibody-dependent cellular capacity in colon cancer using a similar assay in which adherent cells were associated with the cytotoxic effector cell. In our survivors of colorectal cancer, we found a normal range of antibody-dependent cellular activity in two patients, and a low capacity in three. Nevertheless, additional data are needed to ascertain the clinical and prognostic value of antibody-dependent cell-mediated cytotoxicity assays in tumors.

Thus, the cellular immune competence in this particular group of survivors of gastric and colorectal cancers, seems to be preserved. Our findings may suggest that effector immune mechanisms are functionally different in long-term survivors of neoplastic disease than the previously reported altered cell-mediated immunity reactivity in patients with active tumors.

Summary

Cell-mediated immunity was assessed in 12 patients who were long-term survivors of gastric and colorectal adenocarcinomas. A slight decrease in the T-lymphocyte count was accompanied by preserved proliferative reactivity to mitogens (phytohemagglutinin) or alloantigens in 75 percent of the patients. The influence of autologous patient serum on in vitro lymphoproliferative test results was not

significant. Selected sera from both study groups showed values of immune complexes that were within the normal range. The colorectal cancer group had antibody-dependent cellular cytotoxicity within the ranges already established for the normal control subjects. Cellular immune mechanisms seem to have been well preserved in long-term survivors of gastric or colorectal carcinoma.

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