Reprinted from CANCER, Vol. 49, No. 9, May 1, 1982.

Immunology of Human Gastric Cancer:

A Preliminary Report

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The immunological spectrum in fifteen patients with gastric cancer is presented. Patients were divided in three groups. Those with nonadvanced cancer, those with advanced but resectable lesions and those with advanced but nonresectable tumors. Preoperatively, elevated levels of circulating immune complexes (CIC) associated with hyporesponsiveness to phytohemagglutinin (PHA) and in mixed lymphocyte culture (MLC) as well as a positive leukocyte inhibitory serum factor (LIF-S) were found in nearly half of the patients. Inhibitory or enhancing autologous serum factors were detected. Postoperatively, immunologic parameters return to normal in patients with nonadvanced cancer, while in advanced cancer, antibody and cell-mediated immune response remained altered, with some changes associated with chemotherapy. These findings are probably related with the presence or absence of tumor and offer a distinct approach in evaluating the immunologic response of a tumor-bearing patient.

Cancer 49:1810–1816, 1982.

THE INFREQUENT but well-documented spontaneous regression of cancer[1] and the awareness that tumors presumed to have the same histogenesis, degree of differentiation, and clinical stage may behave with varying degrees of aggressiveness,[2] have prompted the search for variables mainly within the field of immunology.[3] In this regard, considerable strides have been made in experimental oncology;[4] in humans, achievements in this field have been piecemeal and subjected to the natural difficulties of a carefully controlled prospective clinical study.[3] In most of the series published, immunologic response is measured by means of one or more parameters that may not only be difficult to integrate but often occur in groups of patients for which a detailed clinipathologic characterization does not exist.

In gastric cancer, the few existing reports have shown some immunological changes in both humoral and cellular immunity.[3,5–9]

In an effort to clarify the immunologic spectrum of human gastric cancer, a prospective study was undertaken in 15 patients which were further subdivided into three groups: (1) those with nonadvanced gastric lesions, (2) those with advanced but resectable tumors, and (3) those with advanced but nonresectable tumors. Patients with benign gastric lesions and normal individuals were included as controls. Data obtained before treatment is compared with that obtained after surgery or during radiotherapy and/or chemotherapy.

Patients

Fifteen patients with gastric cancer (Table 1), of both sexes, aged 36–76 years (40% between 41–50 years) were clinically evaluated in the Gastroenterology Unit of the University Hospital of Caracas, Venezuela. Most of them were admitted for symptoms imputable to the tumor. X-rays studies showed lesions in all cases, although such were not always interpreted as malignancies. Gastroscopy and biopsy were performed in all cases. In 11 instances the lesion was histologically diagnosed as cancer; in one case only severe epithelial atypias could be elicited, and in three the samples showed only chronic gastritis.

All 15 patients were subjected to laparotomy and in ten a gastrectomy (Billroth II) was performed; five cases were considered nonresectable and in three an incisional

Supported by the Venezuelan Public Health Ministry, Central University Scientific Council, Instituto Nacional de Hipódromos, José M. Vargas Foundation and Polar Foundation.

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The authors thank Dr. Ricardo Salomon for his support and Ms. Amanda González H. for her help in the preparation of the manuscrypt.

Accepted for publication March 2, 1981.

0008-543X/82/0501/1810 $0.85 © American Cancer Society

TABLE 1. Histological Grading, Staging and Outcomes for Patients

Case	Histologic Grading Laurén	Staging TNM (Kennedy)	Follow-up
1	Diffuse	T_2-No-Mo	Eleven months postoperative in good condition.
2	Intestinal type	T_2-No-Mo	Chemotherapy. Ten months postoperative, in good condition.
3	Intestinal type	T_1-No-Mo (early cancer)	One year postoperative, free of disease. Suffered heart attack (myocardial infarction).
4	Diffuse	T_3-N_1-M Peritoneum	Chemotherapy. Sixteen months postoperative, in good condition.
5	Combined	T_2-N_2-Mo	Died eleven months postoperative with evidence of metastases.
6	Intestinal type	T_3-N_2-Mo	Chemotherapy and radiotherapy. Died eleven months postoperative with metastases.
7	Diffuse	T_3-N_1-Mo	Chemotherapy. Died nine months postoperative with metastases.
8	Diffuse	T_3-N_1-Mo	Unknown
9	Diffuse	T_3-N_2-Mo	Chemotherapy. Well and free of disease up to three months postoperative. No further control.
10	Intestinal type	T_3-N_2-Mo	Died in immediate postoperative due to septic shock.
11	Combined	T_3-N?-M Greater omentum	Chemotherapy and radiotherapy. Fifteen months postoperative, in good condition.
12	Intestinal type	T_3-N?-M Greater omentum	Chemotherapy. Bone marrow hypoplasia. Sepsis. Appendicitis. Eleven months postoperative, in good condition.
13	Intestinal type*	T_3-N_2-M†	Chemotherapy and radiotherapy. Died 7 months postoperative with metastases.
14	Diffuse*	T_3-N_2-M† Liver-lesser omentum-pelvic peritoneum.	Unknown (discharged in poor condition)
15	Intestinal type*	T_3-N_2-M† Liver-pancreas mesocolon	Unknown (discharged in poor condition)

Group A: nonadvanced cancer (Cases 1 to 3).
Group B: advanced but resectable cancer (Cases 4 to 10).
Group C: advanced nonresectable cancer (Cases 11 to 15).
* Degree of differentiation determined by endoscopic biopsy.
† Metastases appreciated by the surgeon. Unconfirmed histologically chemotherapy: 5-FU in combination with other agents (methyl-CCNU, mitomicyn, methotrexate).

biopsy was conclusive of cancer. The two remaining patients were diagnosed to have cancer only by means of a gastroscopically controlled biopsy. In nine cases, the pathologic study of the resected specimen showed the tumor located in the antrum (in two with extension to the body). The tumors varied in size in between 3–7 cm in maximum dimension, disregarding the sole case with early gastric cancer which measured 1 cm in diameter. The surgical edges proved to be tumor-free in all cases, with the exception of one with residual tumor at the distal end. The tumors were histologically classified for the degree of differentiation according to Laurén.[10] They were properly staged in conformity with the TNM scheme proposed by Kennedy for stomach cancer.[11] All six patients of the control group were subjected to gastrectomy. Four showed a gastric peptic ulcer, one the adult variety of pyloric hypertrophy, and one showed unremarkable changes. This latter case was subjected to surgery as the result of a nonimputable misdiagnosis.

Methods

Blood and sera were obtained from control subjects and from gastric cancer patients at different moments; preoperatively, two or three weeks after surgery, or during radiotherapy and/or chemotherapy.

Preparation of Lymphocytes

Peripheral blood mononuclear lymphocytes (PBL) from cancer patients, benign gastric lesions and normal subjects were isolated by flotation on Ficoll-Hypaque (Winthrop, N. J.) (d-1.077) and washed three times with RPMI 1640 medium (Microbiological Associated, Bethesda, Maryland). The PBL were resuspended at 2×10^6/ml in RPMI 1640 supplemented with 25 mM Hepes buffer, 100IU/ml penicillin-streptomycin (Microbiological Associated, Bethesda, Maryland) and 2% heat-inactivated fresh-frozen, pooled human serum. Prior to the blast transformation test, the PBL were incubated overnight in a humidified atmosphere at

37°C with a 5% CO_2 and air mixture (precultured cells).

Lymphocyte Proliferation Assay (Lp)

Blast transformation to PHA: 2×10^5 mononuclear cells were seeded (in triplicate) on Microtest II plates (Falcon Plastics, Inc., Oxnard, California) in 0.1 ml volume. Mitogen PHA was added at an optimal dose previously determined (5 μg/wells) in 0.1 ml volume.

Transformation to Allogenic Lymphocytes (MLC)

PBL response to alloantigens was examined in one-way MLC, using as stimulator, mitomycin-C-treated (50 μg/ml for 30 min), cryopreserved allogeneic PBL from the standard pool of 17 donors as previously described.[12] Stimulator cells were added at a concentration of 10^5 cell/well to obtain a stimulator–responder ratio of 2:1. The MLC response was performed in the presence or absence of autologous serum, using U bottom Microtest II plates. The microtest plates were covered by rigid lids (No 5 limbro plastics) and incubated in a humidified atmosphere at 37°C with a 5% CO_2 air mixture.

Mitogen cultures were terminated on day four, whereas MLC cultures were harvested on day six, following a 12-hour pulse with 1 μCi of (3H) TdR (New England Nuclear Boston, Massachusettes; specific activity 2 Ci/milimol).

Lymphocyte proliferative response was expressed as a relative proliferation index (RPI) as described by Dean *et al.*[13] RPI is defined as the ratio between the net cpm of the patient and the mean net cpm of three or more normal subjects assayed simultaneously. Cut-off values to define depressed response were established as the bottom ten percentile of normal RPI values.

Leukocyte Migration Inhibition Assay

Leukocyte inhibitory factor activity in serum (LIF-S) was detected using a modified agarose microdroplet assay as described by Weese *et al.*[14] Briefly, 1 ml of leukocytes (2×10^7 cells) were dispensed into a plastic tube and centrifuged at 200 × g for 10 min. Afterward, all medium was carefully removed by a Pasteur pipette, and 0.1 ml of a 2% agarose mixture was added to the leukocytes and the tubes vortexed until a suspension was obtained. Two microliter droplets of this suspension were placed in each well of a flat bottom Microtest II plate with a Drummond microdispenser. After the droplets had solidified for 2–5 min, 0.1 ml aliquots of McCoy's 5 A medium, containing 10% fetal calf serum (Microbiological Associated, Bethesda, Maryland), 25 mM Hepes buffer, and 100 IU/ml penicillin-streptomycin were added to three control wells. Similarly, 0.1 ml aliquots (dilution 1:2 in McCoy's 5 A medium) of each test serum were added to appropriate wells. The plates were covered and kept in a 37°C humidified 5% CO_2 atmosphere for 18 hours.

After incubation each well was projected on to a paper using a photographic amplifier (Omega II); the inner area (the agarose droplet) and the outer area of migration were carefully measured by a manual planimeter. The migration index was calculated by the following formula:

$$MI = \frac{D3D4-d3d4}{D1D2-d1d2}$$

Where D1D2 and d1d2 represent the measure of two diameters (outer and inner migration area, respectively, in the control wells) and D3D4 and d3d4 the outer and inner diameter in the experimental wells. A MI less than 0.80 was taken as inhibitory effect (positive LIF-S).

Circulating Immune Complexes

Detection of circulating immune complexes (CIC) was performed using the Raji cell radioimmunoassay described by Theofilopoulos *et al.*[15] and the I^{125} labeled C1q binding assay (C1q-BA) described by Nydegger *et al.*[16] as modified by Zubler *et al.*[17]

Samples were tested in duplicate and without prior thawing. Those samples greater than 2 S.D. (standard deviations) above the mean of 100 normal controls were considered positive for both tests.

Antibody Dependent Cell Cytotoxicity (ADCC)

The ADCC activity of the patients PBL was determined using Rh-D+ erythrocytes as target cells coated with specific IgG anti-D (Ortho Diagnostic Inc., Raritan, N. J.) 6×10^4 sensitized and 51 Cr labeled erythrocytes were incubated at different ratio with effector cells in 0.2 ml volumen, using a U-bottom Microtest II plate. Plates were incubated at 37°C in an atmosphere of 5% CO_2 air mixture for 18 hours. Results were expressed as the number of mononuclear cells capable of lysing 50% of the sensitized erythrocytes (K): K values were calculated by the Von Krogh equation as modified by Trinchieri *et al.*[18] K values were obtained from 30 blood donors. The normal range calculated as the 80th percentile of those 30 controls was 7.8×10^4–7.8×10^5 mononuclear cells.

Statistical Analysis

The chi-square test was utilized.

TABLE 2. Immunological Parameters Among Populations Studied

Group	N° Patients	Number of cases with elevated levels of CIC depressed response to: Elevated levels of CIC* Raji	Clq-BA	Depressed response to PHA†	MLC†	Positive LIF-S‡	P§
Gastric cancer	15	6/15 (40%)	9/15 (60%)	7/15 (47%)	5/15 (33%)	6/14 (43%)	<0.05
Benign gastric lesions	6	0	0	0	1/6 (17%)	1/5 (20%)	>0.1

* Normal values for CIC (100 cases): Raji ≤ 38 μg equivalent of heat aggregated human gammaglobulin per ml. of serum. Clq-BA ≤ 3.6% of ^{125}I Clq-binding.

† Cut-off values for depressed RPI are based on 70 normal controls for PHA (RPI ≤ 0.65) and 30 normal controls for MLC (RPI ≤ 0.66).

‡ LIF-S was positive in 13% of 24 normal controls assayed simultaneously.

§ Indicate the probability of significance for the difference obtained in comparison with normal controls by chi-squared test.

Results

Preoperative Findings

In Table 2, the results of the immunologic parameters found in both gastric cancer and in the control groups are depicted. In patients with gastric cancer CIC were found to be elevated in 40% (Raji) and 60% (C1q-BA) of the cases. Hyporesponsiveness to PHA and in MLC was found in 47% and 33%, respectively, of the cancer group, while only one control showed a slight decreased response in MLC (RPI: 0.63). Positive LIF-S was demonstrated in 43% of the cancer group, in one of five cases of benign gastric lesions, and in three of 24 normal subjects. The difference between the cancer group and the normal subjects was significant ($P < 0.05$).

The influence of serum factors on *in vitro* cell reactivity (MLC) was sought by cultivating both in normal human serum and in autologous serum. The results are shown in Table 3. Inhibitory or enhancing autologous cancer sera were found on own patient lymphocytes. Further, these effects were also observed on homologous normal lymphocytes.

Postoperative Findings

Only eight of the 15 patients with gastric cancer could be appropriately followed since four died during

TABLE 3. Influence of Cancer Serum Factors on Allogeneic Reactivity of Autologous and Normal Lymphocytes

Case	Responder	MLC response (RPI) in the presence of: Normal human serum	Patient serum	% of inhibition	% of enhancement	Elevated levels of CIC*: Raji	Clq-BA
1 ↓	Patient	0.34	0.11	68		−	+
	N1	0.83	0.16	81			
	N2	1.16	0.44	62			
2 ↓	Patient	1.93	0.44	77		−	−
	N3	1.19	0.53	55			
	N4	0.81	0.27	67			
6 ↓	Patient	0.45	0.004	100		+	+
	N5	1.35	0.006	100			
	N6	1.76	0.012	99			
8 ↓	Patient	0.87	0.11	87		−	−
	N7	1.30	0.02	98			
	N8	0.69	0.49	29			
10 ↑	Patient	1.75	6.02		244	+	ND
	N9	1.22	3.20		162		
	N10	0.77	1.20		56		
11 ↑	Patient	1.08	1.66		54	+	+
	N9	1.22	3.32		172		
	N10	0.77	2.25		192		

RPI = Relative proliferation index; cut-off values defining depressed response, RPI ≤ 0.66.

* Normal values: Raji ≤ 38 μg. equiv. AHGG/ml.; Clq-BA ≤ 3.6% ^{125}I-Clq-binding.

↓ Inhibitory sera.
↑ Enhancing sera.
ND = Not determined.

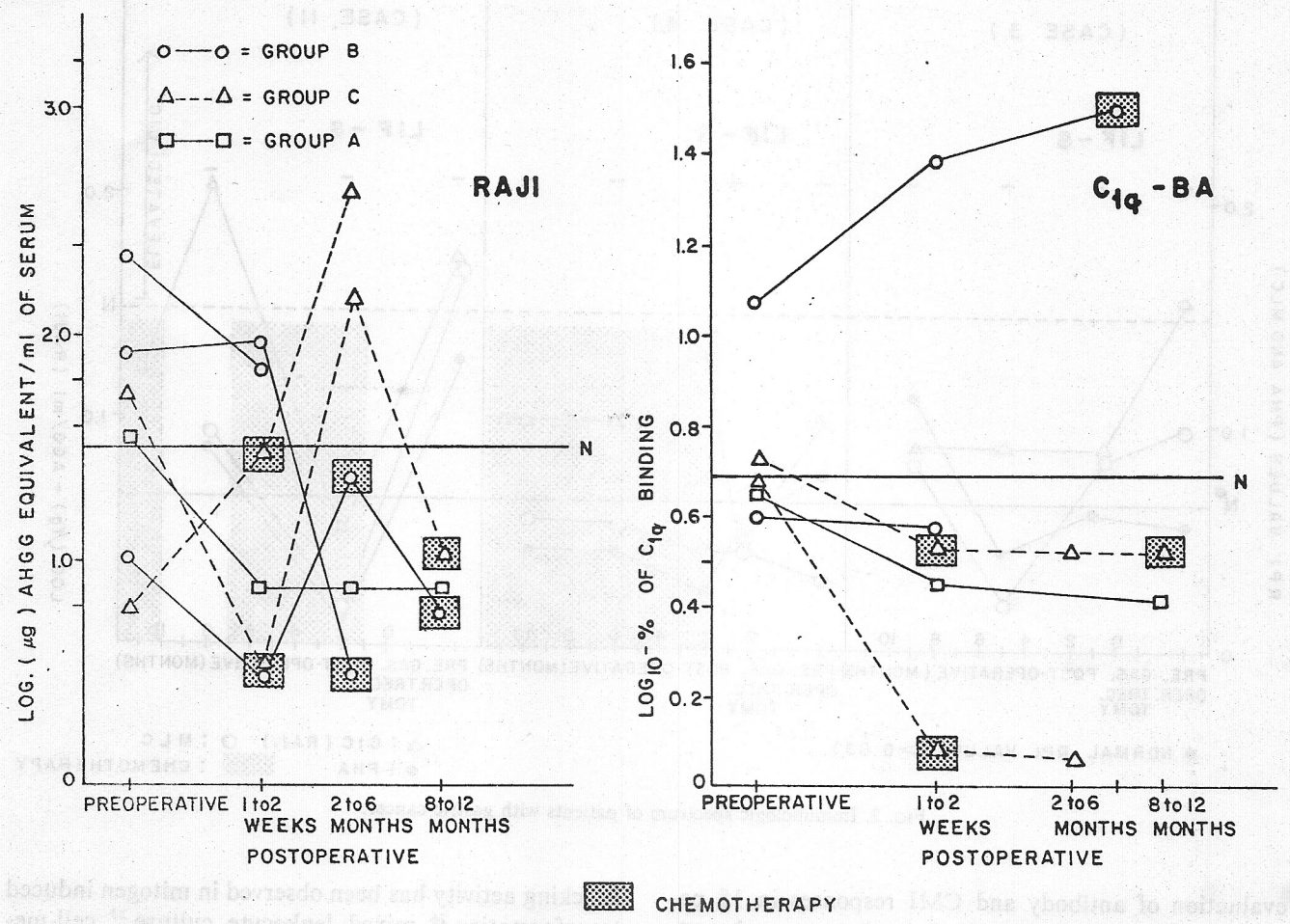

FIG. 1. Postoperative CIC in cancer patients.

hospitalization, two were discharged in poor condition, and one was lost to further follow-up. Of the eight, two belonged to the nonadvanced group (A), four to the advanced but resectable group (B), and two to the advanced nonresectable group (C).

In Figure 1 the results of CIC are depicted. The only patient of Group A showed no elevation of CIC levels (both by Raji and C1q-BA) one year after surgery. Those of Group B with elevated CIC showed a decrease after surgery and chemotherapy by Raji cells, while by C1q-BA no change was noted. Finally, in two cases of group C, CIC were elevated (Raji), decreasing while chemotherapy was given. In regard C1q-BA, the pattern remained unchanged.

In an attempt to integrate antibody and cell mediated immune responses, one cancer patient from each group was selected (Fig. 2). Group A is represented by a patient with early gastric cancer (Case 3). This patient (Fig. 2) ten months postoperative showed normal levels of CIC (Raji), normal responses to PHA and in MLC, as well as a negative LIF-S. During the same period, the patient of Group B (Case 4) had received 12 cycles of chemotherapy. Levels of CIC (Raji) had remained within normal limits while cell responses (PHA and MLC) were depressed. LIF-S was positive two weeks after surgery, becoming negative during chemotherapy. Finally, in the patient of Group C (Case 11) while CIC (Raji) were elevated after chemotherapy, responses to PHA and MLC returned to normal during the same period. LIF-S remained undetectable throughout the follow-up.

Discussion

Very little is known in regard to the immunology of gastric cancer. Positive delayed hypersensitivity skin test to autologous gastric tumor extract has been reported.[13] Theshima *et al.*[6] found elevated levels of CIC by C1q-BA in two cases of gastric cancer. Hyporesponsiveness in blast transformation has also been found in patients with this tumor.[5,8] In the same study, Kamei[8] claimed that leukocyte adherence and migration inhibition may be useful in the diagnosis and recurrence.

Our research protocol has included a simultaneous

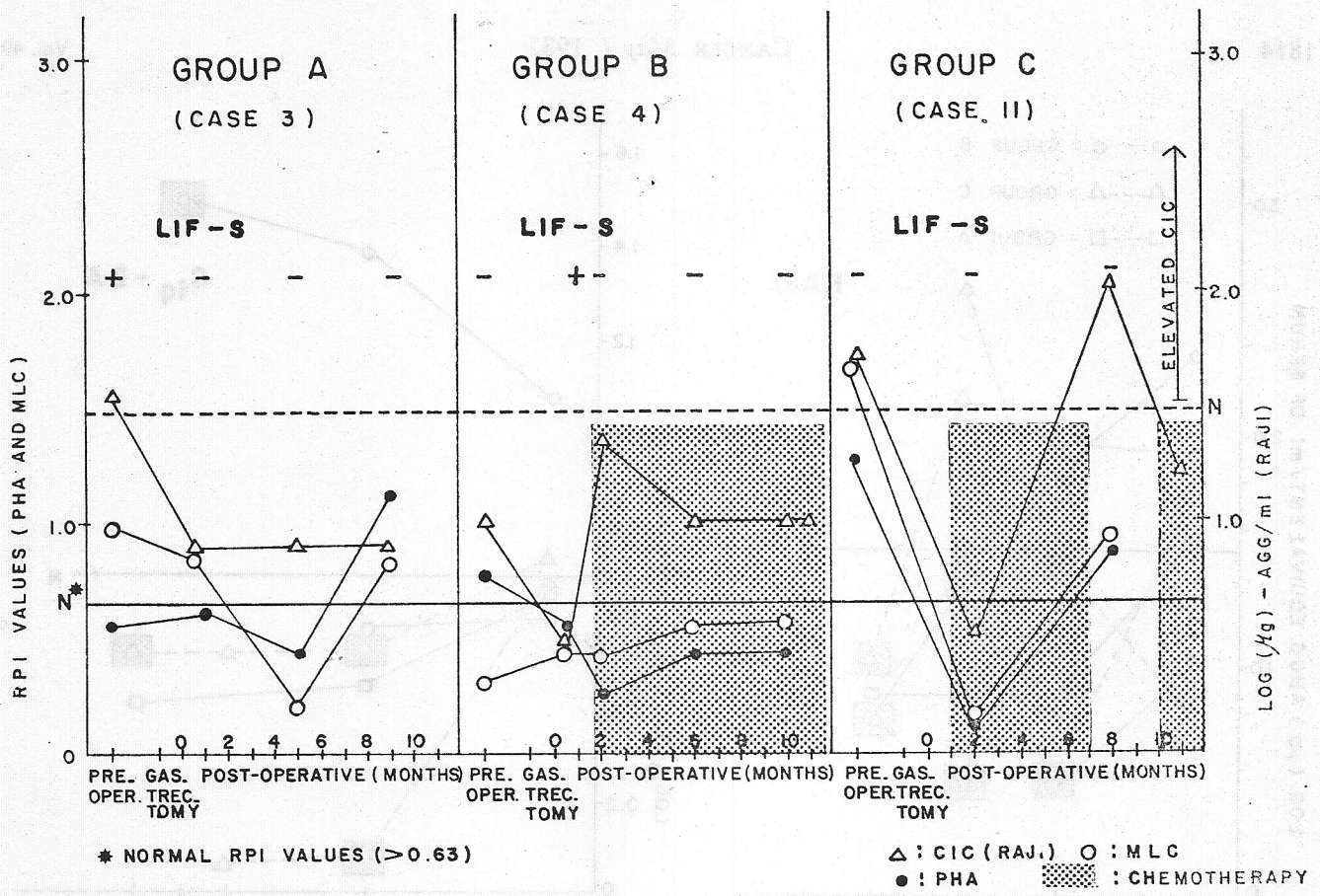

FIG. 2. Immunologic spectrum of patients with gastric cancer.

evaluation of antibody and CMI responses in 15 patients with gastric cancer, both in the pre- and postoperative periods. Preoperative elevated levels of CIC associated with hiporesponsiveness to PHA and in MLC, as well as a positive LIF-S were found in nearly half of the patients, when compared with the control groups.

Hyporesponsiveness in blast transformation to PHA, Con A as well as in unidirectional allogenic reaction has been associated with advanced cancer.[3] However due to the absence of well-standarized methodologic criteria, conflicting results have been reported on the nature and degree of depression of cell reactivity in patients with human cancer.[19-23] On the other hand, numerous reports on CIC in several tumors have shown correlation with extension, recurrence and/or response to chemotherapy.[24,25]

When *in vitro* cell reactivity was explored in the presence of both normal or autologous serum, an inhibitory effect was detected in some of our cases. Many studies have shown that serum from cancer patients commonly produces an abrogating effect on test measuring specific and nonspecific functions of lymphocytes *in vitro*. Blocking activity has been observed in mitogen induced transformation,[26] mixed leukocyte culture,[27] cell-mediated cytotoxicity,[28] the leukocyte migration technique,[29] the leukocyte adherence inhibition assay[30] and antigen stimulated lymphocyte blastogenesis.[31] Nevertheless, in two of our cases an enhancing effect was elicited; this suggests that in patients with gastric cancer, the serum may induce a bimodal effect in *in vitro* cell reactivity. Furthermore, these influences were also observed when normal lymphocytes were utilized.

By performing the agarose microdroplet assay,[14] we have found leukocyte migration inhibitory activity (LIF-S) in the sera of some of our patients; LIF-S was present uniformly in the patients with less advanced cancer and in 50% of those with advanced but resectable tumors, while it was negative in the group with advanced nonresectable tumor. Bruley-Rosset *et al.*[32] reported on the presence of LIF-S in 52 of 99 cases of bronchial carcinoma, 13 out of 49 cases of breast cancer and in 21 out of 45 instances of glioblastoma; furthermore, they found correlations between the detection of LIF-S and the presence of tumor, delayed hypersensitivity reaction to DNCB and recall antigens.

The immunologic data in the postoperative period was most interesting. In Group A, the removal of the tumor by surgery resulted in return to normal of both proliferative responses and levels of CIC in a one-year follow-up. In Groups B and C, proliferative responses were depressed during chemotherapy, returning to normal in some of the patients after finishing each cycle; in the same groups, those with elevated levels of CIC preoperatively showed a normalization by Raji cells after surgery and during chemotherapy, while the pattern of CIC-detected by C1q-BA remained unchanged.

Further, during our research (postoperative period) we standarized a technique for evaluating the ability to mount an antibody-dependent cell-mediated cytotoxicity. Preliminary results are presented in an effort to illustrate the significance of this function in this particular type of cancer. Although we do not know what factors govern the expression of ADCC under the conditions chosen in our protocol, it was remarkable that while in the patient with absence of tumor load and in that subjected to surgery and chemotherapy the ability to mount ADCC was intact ($k = 7.8 \times 10^5$ and 5.2×10^5 respectively), the patient with advanced cancer exhibited an abnormal pattern ($k = 1.5 \times 10^6$) suggestive of lack of ADCC capacity.

A larger series and more prolonged follow-up is essential to further substanciate these preliminary findings. We also suggest that only with the simultaneous estimation of several immunologic parameters of both antibody and CMI response will it be possible to draw a more concrete typification of the tumor-bearing patient when first diagnosed. This approach offers a most convenient mean to evaluate not only the effectiveness of conventional or new forms of therapy but might also reveal undesirable influences of these several forms of therapy on the immune responses of a tumor-bearing host.

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