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IMMUNE RESPONSES IN EC PATIENTS

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ABSTRACT

General immune responses were studied in patients with esophageal carcinoma (EC) and were compared to those in normal individuals of similar age, sex and ethnic origin. There was a significant increase in the titer of "background" antibodies in the sera of cancer patients; however, this elevation was not associated with an increase in the levels of serum immunoglobulins. EC patients had a diminished number of E rosetting lymphocytes but normal proportion of EAC rosetting cells. Lymphocytes from a small but significant number of patients showed decreased responses to PHA when cultured in medium containing fetal calf serum. Plasma samples from these patients were capable of inhibiting the *in vitro* proliferative responses of lymphocytes to PPD.

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INTRODUCTION

A number of studies have revealed varying levels of immunoincompetence in patients with malignant diseases of nonlymphoid tissue (Garrioch *et al.*, 1970; Ducos *et al.*, 1970; Whittaker *et al.*, 1971; Catalona *et al.*, 1973; Knight and Davidson, 1975; Chan *et al.*, 1976; Cochran *et al.*, 1976). In this paper, we present information on the immunological state of patients with esophageal carcinoma (EC). The immunological competence of the patients was assessed prior to any therapy by determination of serum immunoglobulin levels, titers of "background" antibodies to ubiquitous antigens, levels of E and EAC rosetting lymphocytes in the peripheral blood and by the in vitro lymphocytes responsiveness to phytohemagglutinin (PHA). In addition, the effect of plasma from these patients on the *in vitro* reactivity of normal lymphocytes to PPD was determined.

MATERIALS AND METHODS

Patients

The present study is one of a series characterizing the esophageal cancer patients and the population at high risk. One hundred and ten esophgeal cancer patients (76 male and 34 females) seen at Pahlavi Hospital in Gorgan as well as those who came to Teheran for treatment were studied preoperatively. They ranged in age from 29 to 76 years, and all had a positive diagnosis of cancer based on clinical criteria as well as radiology, endoscopy and biopsy.

Control data were obtained by examining 137 healthy individuals (94 males, 43 females) from Gorgan or the surrounding villages. The age distribution of the controls ranged from 20 to 77 years. Based on the availability of samples, some or all of the tests were performed on each individual.

Assessment of general immunological competence

The level of immunoglobulins (IgM, IgG, and IgA) was estimated using single radial immunodiffusion (Mancini *et al.*, 1965) on commercially prepared plates (Hoechst Pharmaceuticals). Titer of "background" antibodies to bovine serum albumin (BSA), egg albumin (EA), keyhole limpet hemocyanin (KLH), *Entameba histolytica* antigen (EH) and sheep red blood cells (SRBC) was measured by

passive hemagglutination using chromic chloride-treated erythrocytes (Faulk and Houba, 1973) and by hemagglutination. Titers are expressed as the $\log_2$ of the highest dilution showing agglutination.

The percentage of E and EAC rosetting lymphocytes in peripheral blood was estimated employing the method of Jondal *et al.* (1972). In each test 200 lymphocytes were scored and those cells binding three or more sheep red blood cells were considered rosettes.

T-cell function was studied by assessing the lymphocyte response to phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, MI). Peripheral blood leukocytes were separated by Ficoll-Hypaque centrifugation as described by Boyum (1968), and cells from the interface layer were washed three times in TC 199 medium. Cells were then suspended in TC 199 supplemented with 10% fetal calf serum, 1% TC glutamine, 100 IU/ml penicillin, 100 ug/ml streptomycin and 100 ug/ml kanamycin. The pH of this medium was adjusted to 7.3 $\pm$ 0.2 using 7.5% sodium bicarbonate. Assessement of cell viability was performed by the trypan blue exclusion technique. By this criterion, 95-99% cells were viable. Cell concentration was then adjusted to 3 x 10^5 per ml medium. For stimulation, 0.1 ml of a 1:64 or 1:128 dilution of PHA was added to each of the four culture tubes containing 0.9 ml cell suspension. For control purposes 0.1 ml of medium was added to another four tubes. The cells (1 ml/tube) were then cultured at 37°C for 72 hours in a humid atmosphere containing 5% CO_2. Sixteen hours before termination of the culture, 1 uCi-tritiated thymidine (specific activity 21.5 Ci/mM) (Radio Chemical Center, Amersham, England) was added to each culture tube. The cell pellets were collected by centrifugation at 700 xg for 10 min. Incorporation of ^{3}H-thymidine was stopped by addition of 5 ml 0.14 M ice-cold PBS (pH = 7.2). The cells were then washed three times with phosphate buffered saline (PBS) and the acid-insoluble fraction was precipitated with 5 ml of ice-cold trichloroacetic acid at 4°C. After allowing 30 minutes for precipitation to take place, tubes were centrifuged at 700 xg. The precipitates were washed in 5 ml absolute methanol, then left to dry in inverted position at 37°C. When dried, precipitates were dissolved in 0.4 ml soluene-100 (Packard Instruments, Inc., Downers Grove, IL). Solubilization was achieved by incubating the tubes for 2 hours at 56°C. Each preparation was then transferred to scintillation vial containing 3 ml scintillation fluid (Packard Instruments). Radioactivity in each sample was counted in a Tri-Carb liquid scintillation spectrometer. The results are expressed by stimulation indices (SI), computed as:

$$SI = \frac{\text{CPM of lymphocytes stimulated by PHA}}{\text{CPM of unstimulated lymphocytes}}$$

CPM corresponds to the mean count per minute of each quadruplicate culture after correction for the CPM of medium control.

Effect of plasma for EC patients on the lymphoproliferative response was determined by comparing the response of lymphocytes to PPD in cultures containing 15% patient plasma with parallel cultures containing pooled normal AB plasma, kindly provided by the Iranian Blood Transfusion Service. For stimulation 4×10^5 cells from a single normal donor were cultured with 10 and 100 ug PPD (Statens Seruministute, Copenhagen, Denmark) for 72 hours. Control cultures were incubated in the absence of PPD. Cultures were then pulsed with 1 uCi of ^{3}H-thymidine, incubated for an additional 18 hours, then harvested according to the procedure detailed above The stimulation indices were calculated as follows:

$$SI = \frac{\text{CPM/of cells cultured with PPD in the presence of patients or AB plasm}}{\text{CPM/of cells cultured in the absence of PPD}}$$

RESULTS

Serum levels of IgM, IgG, and IgA were similar in the esophageal cancer patients and the controls (Table I). Cancer patients, however, had significantly elevated levels of "background" antibodies with the exception of antibody to SRBC which was comparable in both groups.

The percentage of EAC rosetting lymphocytes was comparable in EC patients and normal individuals of similar age ($33 \pm 9\%$, vs $31 \pm 8\%$) (Table III). Lymphocytes forming E rosettes, however, were marginally less frequent in peripheral blood of the cancer patients than in the controls ($P < 0.05$ by "one-tailed" Student t-test).

The response of lymphocytes from 38 EC patients and 25 normal individuals of the same age range are compared in Table IV and graphically presented in Figure 1. Data presented here were obtained using a 1:128 dilution of PHA at which maximum stimulation occurred. Considerable variation in the range of SI was observed in both groups. However, a small but significant percentage (32%) of EC patients responded poorly to mitogen, producing low stimulation indices (values between 1.1-20). Such low values, however, were not encountered among the normal individuals, and this difference is significant at $P = 0.0037$ by Fishers exact test.

Table IV shows the effect of plasma from 17 EC patients on

the ability of lymphocytes from a single donor to produce a proliferative response to PPD. Without exception, addition of patient plasma to lymphocyte cultures resulted in a significant suppression of PPD response as compared to cultures containing pooled normal AB plasma.

DISCUSSION

The present study compared the general immune status of esophageal cancer patients with that of normal individuals having similar age, sex and ethnic composition. In general, there was a significant increase in the titer of "background" antibodies in the sera of EC patients as compared to the normal controls. Included in these studies of antibody were anti-BSA, anti-EA, anti-KLH, anti-EH and anti SRBC. However, elevation in the titer of antibodies was not associated with a significant increase in the levels of serum immunoglobulins, or with a deviation from normal in the percentage of EAC rosetting lymphocytes, comprising the B cell population. EC patients, however, had a diminished number of T cells (E rosetting lymphocytes), and lymphocytes from a significant proportion of the patients (32%) showed decreased responses to PHA (a T cell mitogen) when cultured in medium containing fetal calf serum. In addition, plasma samples from EC patients were capable of inhibiting the *in vitro* proliferative response of lymphocytes to PPD.

Our findings regarding low lymphocyte responsiveness to mitogen, as well as reduction in the relative proportion of lymphocyte subpopulations in patients with esophageal carcinoma, confirm a number of prior observations (Ducos *et al.*, 1970; Whittaker *et al.*, 1971; Knight and Davidson, 1975; Chan *et al.*, 1976). Presence of inhibitor in the plasma of EC patients was not surprising, since suppression of lymphocyte functions *in vitro* by humoral factors present in the plasma of cancer patients has been repeatedly well-documented (e.g., Sample *et al.*, 1971; Succui Foca *et al.*, 1973; Vankey *et al.*, 1971 and 1975). The diminution in lymphocyte responsiveness to PHA noted in some EC patients could be the reflection of the tumor burden or, alternatively, could be the consequence of poor health and nutritional status of the patients.

Our findings concerning increased levels of "background" antibody in the EC patients are inconsistent with those of Parfentjev *et al.* (1951) who observed a decline in the incidence of natural agglutinin for proteus in patients with untreated malignancies. A

possible link between depression of T cell function and high "background" antibody levels in the sera of patients with esophageal carcinoma remains to be established. It is possible that the tumor somehow commits B cells to nonspecific antibody production. Alternatively, the tumor may bring about an alteration in the control of B cell activity.

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LEGEND

Figure 1. Comparison of lymphocyte response to PHA (1:128) in EC patients and normal controls. Each point represents one individual.

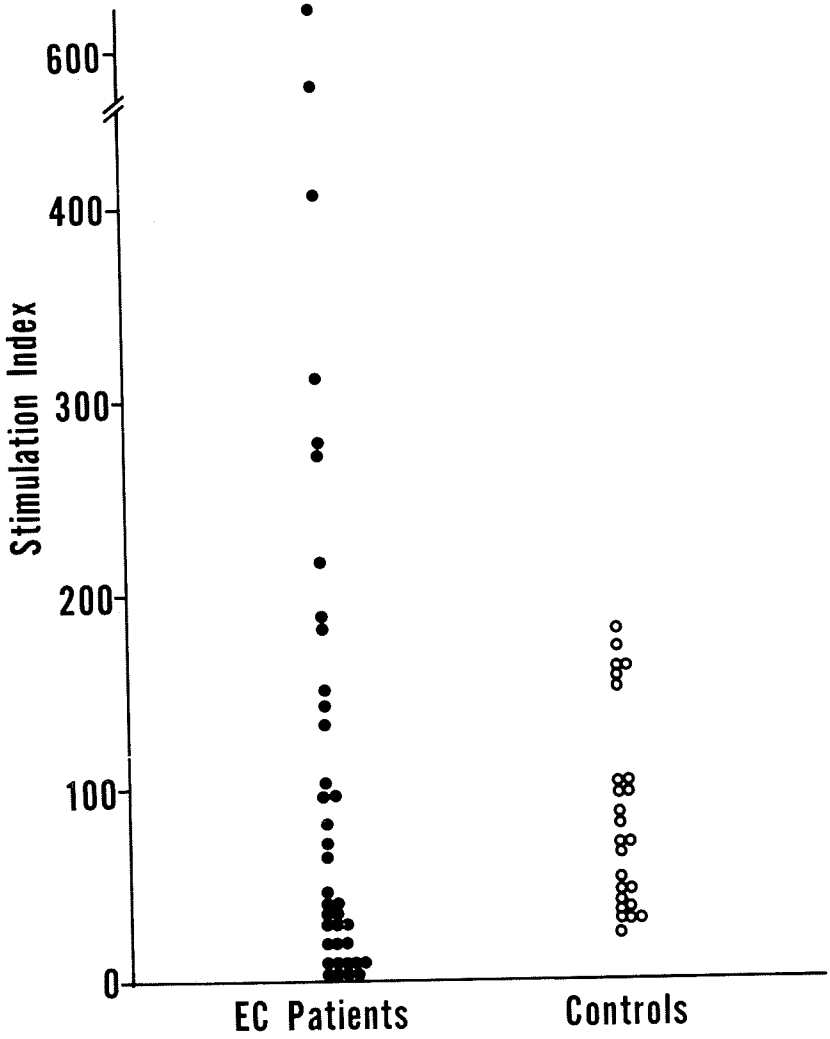

TABLE I

COMPARISON OF IMMUNOGLOBULIN LEVELS IN EC PATIENTS AND NORMAL CONTROLS

Age		Mean serum immunoglobulin levels ± S.E. (mg/100 ml) EC	Non EC	t[1]	df[2]
30-39	IgM	200± 83	170± 86	1.08	36
	IgA	416± 96	265±100	1.57	36
	IgG	1914±430	2023±365	-0.82	34
40-49	IgM	180± 80	164± 77	0.83	70
	IgA	279± 95	293± 97	-0.53	70
	IgG	1731±738	1901±331	-1.14	68
50-59	IgM	170± 95	197± 90	-1.17	65
	IgA	290± 92	294± 89	-0.22	64
	IgG	1895±471	1777± 39	1.11	63
60-69	IgM	161± 97	165± 68	-0.12	29
	IgA	336±102	301±104	0.94	29
	IgG	2081±400	1718±586	1.84	24
70+	IgM	177± 57	178± 91	-0.03	14
	IgA	428±252	270± 80	1.69	14
	IgG	2086±699	1616±259	1.79	14

[1] t value

[2] degree of freedom

TABLE II

COMPARISON OF "BACKGROUND" ANTIBODY TITERS IN EC PATIENTS AND NORMAL CONTROLS[1]

Age	Disease Status	Mean antibody titers (log_2) ± S.E.				
		Anti-KLH	Anti-EA	Anti-SRBC	Anti-EH	Anti-BSA
20-29	EC (4)[2]	6.98±2.63	4.48±1.57	3.48±0.53	3.30±0	4.48±1.95
	Non EC (28)	4.20±1.40	3.34±0.65	3.38±0.48	3.26±0.35	3.24±0.28
30-39	EC (16)	5.49±1.46	4.22±1.45	3.35±0.39	3.80±0.67	3.77±0.81
	Non EC (32)	4.20±1.30	3.29±0.76	3.26±0.59	3.47±0.63	3.35±1.03
40-49	EC (28)	5.15±2.93	4.56±1.98	3.32±0.40	3.85±1.06	3.85±0.81
	Non EC (35)	4.11±1.29	3.34±0.49	3.22±0.37	3.56±1.07	3.22±0.31
50-59	EC (27)	4.66±1.40	3.96±0.95	3.25±0.45	3.36±0.65	3.59±0.51
	Non EC (20)	4.03±0.90	3.24±0.73	3.11±0	3.30±0.36	3.06±0.12
60-69	EC (17)	4.56±1.30	3.95±1.50	3.22±0.17	4.53±2.21	3.26±0.36
	Non EC (8)	4.02±0.89	3.98±1.46	3.42±0.53	3.58±0.97	4.13±1.71
70 +	EC (12)	4.90±1.88	3.87±0.96	3.46±0.75	3.80±1.03	3.76±1.03
	Non EC (8)	3.57±0.78	3.40±0.78	3.33±0.86	3.15±0.17	3.12±0
Mean difference in titer EC - Non EC		1.27	0.75	0.06	0.40	0.50
	t[3]	5.71	5.52	1.18	2.10	4.79
	p	< 0.001	< 0.001	> 0.05	< 0.05	< 0.001

[1] Titers are expressed as the log_2 of the highest dilution showing agglutination.

[2] Number of samples tested.

[3] The t value given in the table corresponds to the F statistic on 1 and n degrees of freedom corresponding to the esophageal cancer-non-esophageal cancer main effect in a full analysis of variance taking account of both disease status and age.

TABLE III

COMPARISON OF LYMPHOCYTE SUBPOPULATION IN EC PATIENTS AND NORMAL CONTROLS[1]

Disease status	Number tested	% EAC rosetting lymphocytes	% E rosetting lymphocytes
EC	28	33 ± 9	56 ± 11[2]
Non EC	31	31 ± 8	60 ± 6

[1] Results are expressed as means ± SD.

[2] $P < 0.05$ by "one-tail" Student t test.

TABLE IV

COMPARISON OF LYMPHOCYTE RESPONSE TO PHA IN EC PATIENTS AND NORMAL CONTROLS[1]

Disease Status	Stimulation Index		
	≥40	≤20 <40	<20
EC	19	9	10
Non EC	18	7	0

[1] $P = 0.0037$ by Fishers exact test.

TABLE V

EFFECT OF PLASMA FROM EC PATIENTS ON THE IN VITRO PPD RESPONSE[1]

Expt. No.	Plasma Donor[2]	Stimulation Index[3] μg PPD 100	10
1	AB	22.0	18.0
	P_5	8.8	10.2[4]
	P_6	10.4	2.8
	P_8	11.6	12.1[4]
	P_9	8.0	11.7[4]
	P_{10}	9.9	7.9
2	AB	4.4	7.5
	P_1	3.1	4.9
	P_3	1.5	4.1
	P_{15}	1.7	3.0
	P_{16}	1.9	4.0
	P_{17}	3.0	4.0
	P_{21}	3.0	3.9
	P_{22}	1.9	4.4
	P_{23}	2.6	2.9
	P_{24}	2.6	3.8
	P_{25}	1.6	3.5
	P_{26}	1.6	2.1
	P_{27}	2.7	3.8

[1] 15% plasma was used.

[2] AB, normal pooled AB plasma; P, patient's plasma

[3] Cells from a single donor were used. SI values differ significantly from the control of each experiment at $P < 0.01$.

[4] $P < 0.05$.

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