Cell-Mediated Immunity in Patients with Invasive Carcinoma of the Cervix

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Multiple in vitro immune parameters were investigated in thirty-four untreated patients with invasive carcinoma of the cervix and in twenty-five controls. The parameters measured were percentages and absolute counts of T and B cells, percentage of T cell subsets, lymphocyte response to phytohemagglutinin (PHA) and concanavalin A (Con A), natural killer (NK) activity, antibody-dependent cellular cytotoxicity (ADCC), and interleukin 2 (IL-2) activity. Patients with invasive cervical carcinoma, as compared with controls, showed a decrease in the percentage and count of T cells, a decrease in the percentage of helper-inducer (CD4+) T cells, decreased CD4+/CD8+ ratio, depressed lymphocyte response to PHA and Con A, and depressed NK and ADCC activities. There were no significant differences in these immune parameters between early and advanced tumor stages. The levels of total lymphocytes, monocytes, suppressor-effector (CD8+) T cells, and B cells were similar to those of the controls. IL-2 productivity in patients was lower than that in controls. In patients with invasive cervical carcinoma, a decrease in the percentage of CD4+ cells was associated with depressed PHA response and decreased IL-2 productivity was correlated with the reduced percentage of CD4+ cells and decreased NK activity. This study shows a significant defect in an important immune surveillance mechanism in patients with invasive carcinoma of the cervix and suggests that impaired IL-2 activity production may be related to quantitative and qualitative alterations in lymphocyte subpopulations which play a major role in immune surveillance against cervical cancer.

Key Words: Cervix, carcinoma, cell-mediated immunity.

The development and course of certain neoplasia are frequently correlated with immunological mechanisms (Burnett 1970; Gatti and Good 1971; Penn and Starzl 1972; Robert and Bell 1976; Ishiguro et al. 1980). It has been reported that the more competent and functioning the immune responses are, the better will be the patient's response to treatment and the longer will be the survival (Lundy et al. 1974; Rafla et al. 1978; Vilien et al. 1981; Shimokawara et al. 1982; Kabawet et al. 1983). The immunologic monitoring of cancer patients is deemed to be of great practical clinical importance. Since Levi (1971) demonstrated tumor-associated antigens (TAAS) in cervical carcinoma tissue by precipitin tests, there have been many reports that cervical carcinoma cells possess

TAAS (DiSaia et al. 1975; Bigbee and Jensen 1978; Goldenber et al. 1978; Kjorstad and Orjasetter 1978; Cocchiara et al. 1980; Kato et al. 1983) and that immune reactions can be generated against these TAAS (Chen et al. 1975; Chiang et al. 1976; Hakala et al. 1976; McCoy et al. 1983). The essential part in tumor defense involves, most probably, the cell-mediated branch of the immune system (Burnett 1970; Gatti and Good 1971; Lander and Aherne 1972; Heberman 1973).Studies evaluating cell-mediated immunity in cervical carcinoma have been reported, yielding conflicting results according to the authors or methods (DiSaia et al. 1971, 1972; Hagen et al. 1972; Chen et al. 1975; Bigbee and Jensen 1978; Goldenber et al. 1978; Kjorstad and Orjasetter 1978; Cocchiara et al. 1980; Stam et al. 1981; Kato et al. 1983; Kietlinska 1984). There have been only a few studies regarding general immune reactivity in patients with cervica carcinoma by measuring multiple immune parameters especially interleukin-2 which plays a major role in immune reactions against tumors (Yamamoto 1986).

In the present study, we attempted to investigate the following: (1) the general immune status in pa-

tients with invasive cervical carcinoma by measuring percentage and count of lymphocytes, monocytes, T and B cells, percentage of T cell subsets, lymphocyte response to phytohemagglutinin (PHA) and concanavalin A (Con A), natural killer (NK) cytotoxicity, antibody-dependent cell mediated cytotoxicity (ADCC), and interleukin 2 (IL-2); (2) the relationship between the clinical tumor stage and the immunocompetence of patients; and (3) the relationship between various immunological parameters.

MATERIALS AND METHODS

Patients and Controls

Thirty-four patients with biopsy-proven invasive carcinoma of the cervix treated at the Department of Obstetrics and Gynecology, Yonsei University College of Medicine were investigated before treatment. The staging was done according to FIGO classification (1982), and the patients were divided into early stages (stage I and stage II) and advanced stages (stages III and IV). Twenty-five normal healthy women matched for age and marital status with the patients were used as controls. Patients excluded from the evaluation included those who had concomitant serious illness, viral infection, use of medication, ad previous or active treatment including surgery, radiotherapy, or chemotherapy. Statistical analysis was carried out using Student's t test to compare mean values of immune parameters in patients and normal controls. Relationships between immune parameters in patients were studied using regression analysis. A result was considered significant if the p value was less than 0.05.

Preparation of Lymphocytes

Peripheral blood mononuclear cells (MNC) were separated by sedimentation in Ficoll-Hypaque (density 1.077 g/ml, Pharmacia) according to the method described by Böyum (1968). Seven ml and 5 ml of heparinized blood were carefully layered on an equal part of Ficoll-Hypaque in each of two 15 ml and one 10 ml centrifuge tubes. The tubes were centrifuged for 30 min at 18-22°C and 400xg. The upper supernatant fluid (plasma and platelet cell layer) was carefully aspirated and discarded and the white interface containing the mononuclear cells was transferred to a 10 ml glass tube. The separated MNCs were adjusted to the desired concentration in RPMI 1640 medium buffered with 25 mM HEPES, containing 4 mM glutamine and antibiotics (100 units/ml streptomycin, 100 units/ml penicillin), supplemented with 10% fetal calf serum.

Total T and B Lymphocyte Population

The T lymphocyte population was determined by its ability to form colorless rosettes, whereas the percentage of B lymphocytes was assessed by their ability to form yellow-brown rosettes using the Quantigen T and B cell assay kit (BioRad). The MNC concentration was adjusted to 1×10^7 cells/ml using a hemocytometer and 100 µl of the cell suspension was added to a siliconized glass tube. Two hundred µl of the resuspended Immunobead Reagent was gently mixed with the cell suspension and centrifuged for 3 min at 150 × g. After incubation at 37°C for 30 min, 100 µl of the vital stain (erythrosin B) was added to the pellet and the pellet was resuspended. A drop of the sample was transferred to a clean microscope slide and at least 200 live lymphocytes were counted using a 40X objective. Rosettes were counted as positive if three or more beads were attached to a cell. Absolute numbers of the T and B cells were calculated from the total lymphocyte counts.

T Lymphocyte Subsets

T lymphocyte subsets were assessed by their ability to form rosettes using the Quantigen T4/T8 cell surface marker assay kit (BioRad). The CD4+ cell was identified by its ability to form red beads while the CD8+ cell was identified by its ability to form yellow-brown beads on its surface. The procedure was performed in the same way as that described in the T and B lymphocyte assay.

Blastogenic Response to PHA and Con A

Triplicate cultures with 2×10^6 MNC per well of the microtest plate (Costar) were incubated with or without PHA (2 µg/ml) or Con A (2 µg/ml) for 72 hours in humidified 5% CO_2 atmosphere at 37°C. Eighteen hours before harvesting, 1 µCi of ^{3}[H]-thymidine (specific activity 20.0 Ci/mM, NEN) was added to each culture. The ^{3}[H]-thymidine incorporation by the cells of each well was assessed on a liquid scintillation counter. The results were expressed as stimulation index, as obtained by the following formulation:

$$\text{Stimulation Index} = \frac{\text{maximum 3[H]-thymidine uptake}}{\text{spontaneous 3[H]-thymidine uptake}}$$

NK Cytotoxicity

The erythroleukemic cells, K562 cells (provided by the Dept. of Microbiology, Yonsei University College of Medicine (YUMC)) were used as target cells. One

million viable K562 cells in the growth phase were labeled by the addition of 100 μCi of sodium chromate (Na$_2$^{51}CrO$_4$, specific activity 1 mCi/ml, NEN) and incubated for 1 hour at 37°C in a 5% CO$_2$ atmosphere. The target cells were then washed three times, resuspended at a concentration of 1 × 10^5 cells/ml, and placed into round-bottomed microwells with 10^4 cells per well in 0.1 ml culture media. The MNCs (effector cells) were then washed and the concentration was adjusted to 5 × 10^6 cells/ml in culture media. The effector cells were added to the K562 target cells at an effector to target ratio of 50 in triplicate and the effector-target mixtures were subsequently incubated at 37 °C in a 5% CO$_2$ incubator for 4 hours prior to testing. As a control, the target cells were incubated in culture media in the absence of effector cells. The percentage of cytotoxicity for each replicate was calculated from the following formula to find out the mean percent cytotoxicity:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum uptake} - \text{spontaneous release}} \times 100$$

Experiemtnal release of sodium chromate was determined by incubating effector cells mixed with target cells. Spontaneous release of sodium chromate was determined by incubating target cells in the absence of effector cells. Maximum release was determined by incubating target cells in 10% phenoxypolyethanol (triton X-100).

Antibody-Dependent Cellular Cytotoxicity

One million L1210 cells (provided by the Dept. of Microbiology, YUMC) were labeled with 100 μCi of sodium chromate and resuspended at a concentration of 1 × 10^5 cells/ml. In 1 × 10^5 target cells, 50 μl of a 1:1000 dilution of anti-L1210 rabbit serum and 5 × 10^6 effector cells were added. Incubation,

harvesting, and calculation of specific chromium release were carried out by the method described for NK cytotoxicity.

Interleukin 2 (IL-2)

IL-2 production: Five million MNCs were suspended in 1 ml of RPMI 1640 medium supplemented with 2 μl PHA, and cultured at 37°C for for 24 hours in a 5% CO$_2$ atmosphere. At the end of the culture period, the cells were removed by centrifugation and the supernatant was collected and stored at −4°C until use.

Assay for IL-2 activity: Fifty thousand (100 μl) CTLL2 (provided by the Dept. of Microbiology, YUMC) cells were added to 100 μl of supernatant to be tested. Assays were carried out in 96-well microplates which were incubated at 37°C in a 5% CO$_2$ atmosphere for an additional 24 hrs. One μCi of ^{3}[H]-thymidine was added to each well and incubated for 4 hours. Cultures were harvested followed by determination of ^{3}[H]-thymidine uptake.

RESULTS

Percentage and count of total lymphocytes and monocytes in the peripheral blood of patients with untreated invasive cervical cancer and normal controls are presented in Table 1. There were no significant differences between both groups in the percentage and count of total lymphocytes and monocytes. Table 2 shows the mean values of count and percentage of T and B lymphocytes in the peripheral blood of untreated cervical carcinoma patients and controls. Mean count and percentage of T lymphocytes was significantly lower in patients with cancer (1554±612/cu mm and 61.5±10.8%, respectively) than in controls (1750±574/cu mm and 72.4±8.2%, respectively) ($p < 0.05$), whereas the count

Table 1. Count and percentage of total lymphocytes and monocytes in peripheral blood of patients with invasive cervical carcinoma and controls

	Lymphocytes		Monocytes	
	Count (/ cu mm)	%	Count (/ cu mm)	%
Patients (34)*	1976±1280	36.4±8.9	491±262	6.5±2.5
Controls (25)	2210± 625	38.0±5.2	542±271	7.4±2.8

Data are expressed as Mean ± SD.

All differences are not significant.

* The number in parenthesis is the number of subjects tested.

Table 2. Count and percentage of lymphocyte subpopulations in peripheral blood of patients with invasive cervical carcinoma and controls

	T Cells		B Cells	
	Count (/ cu mm)	%	Count (/ cu mm)	%
Patients (34)**	1554±612*	61.5±10.8*	263±113	10.6±4.8
Controls (25)	1750±574	72.4± 8.2	288± 87	12.4±4.8

Data are expressed as Mean ± SD.

* p<0.05 as compared to controls.

** The number in parenthesis is the number of subjects tested.

Table 3. Percentage of T cell subpopulations of peripheral blood lymphocytes from patients with invasive cervical carcinoma and controls

	% of cells reactive to monoclonal antibody		CD4+/CD8+
	CD4	CD8	
Patients (34)**	36.3±10.1*	26.0±8.0	1.55±0.56*
Controls (25)	45.6± 8.9	23.8±6.6	2.09±0.75

Data are expressed as Mean ± SD.

* p<0.05 as compared to controls.

** The number in parenthesis is the number of subjects tested.

Table 4. Response to plant mitogens of peripheral blood lymphocytes from patients with invasive cervical carcinoma and controls

	Mitogen	
	PHA (2μg/ml)	Con A (2 μg/ml)
Patients (34)**	7.8± 5.4*	6.9±8.0*
Controls (25)	22.7±14.7	12.3±7.8

Stimulation index is expressed as Mean±SD.

* p<0.05 as compared to controls.

** The number in parenthesis is the number of subjects tested.

Table 5. NK cytotoxicity and ADCC of peripheral blood lymphocytes from patients with invasive cervical carcinoma and controls

	% specific cytotoxicity†	
	NK activity	ADCC
Patients (34)**	25.8±16.5*	50.8±13.0*
Controls (25)	40.5±14.7	61.8±10.5

Data are expressed as Mean ± SD.

† Effector : Target = 50 : 1

* p<0.05 as compared to controls.

** The number in parenthesis is the number of subjects tested.

and percentage of B lymphocytes appeared to be slightly lower than that of normal controls, but this depression was not statistically significant.

The percentage of CD4+ and CD8+ lymphocytes, representing helper-inducer and suppressor-cytotoxic cells respectively, from untreated cervical carcinoma and controls are shown in Table 3. The percentage of CD4+ cells in cancer patients (36.3±10.1%) was significantly lower than in controls (45.6±8.9%, p<0.05). Although a slight increase was noted in the mean percentage of CD8+ cells in patients with cervical carcinoma (26.0±8.0%) when compared with controls (23.8±6.6%), the difference between the means was not significant. A CD4+/CD8+ ratio of 1.55±0.56 was found for cervical carcinoma patients, whereas the control value was 2.09±0.75 and the difference was statistically significant.

The stimulation index of lymphocyte response to PHA and Con A was significantly lower in cancer patients (7.8±5.4 and 6.9±8.0, respectively) than in controls (22.7±14.7 and 12.3±7.8, respectively) (Table 4). Table 5 shows that NK and ADCC activities in cervical cancer patients (25.8±16.5% and 50.8±13.0%, respec-

tively) were lower than those of controls (40.5±14.7% and 61.8±10.5%, respectively).

The mean values for IL-2 productivity determined by shortterm ³[H]-thymidine uptake assay were lower in the patient group than in controls (5.0 units/ml versus 7.3 units/ml) (Fig. 1).

Table 6 shows the immune parameters of patients with invasive cervical cancer according to the tumor
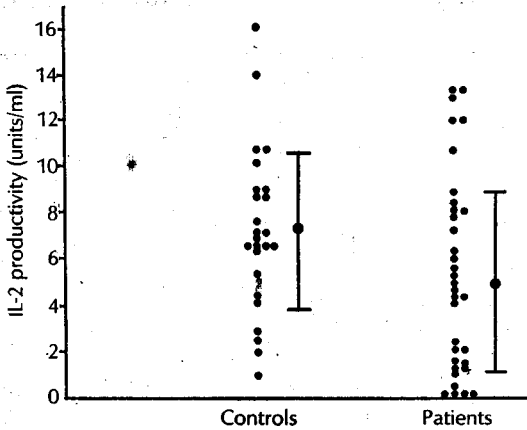
Fig. 1. Interleukin 2 (IL-2) productivity of peripheral blood lymphcytes from patients with invasive cervical carcinoma (5.0±4.1 units/ml) and controls (7.3±3.2 units/ml) (p<0.05).

Fig. 2. Relationship between T helper cell ratio(%) and lymphocyte response to PHA of peripheral blood lymphocytes from patients with invasive cervical carcinoma (linear regression: y=0.19x + 0.84, r-square=0.13, p<0.05).

Table 6. Comparison between the clinical tumor stage and measured immune parameters in patients with invasive cervical carcinoma
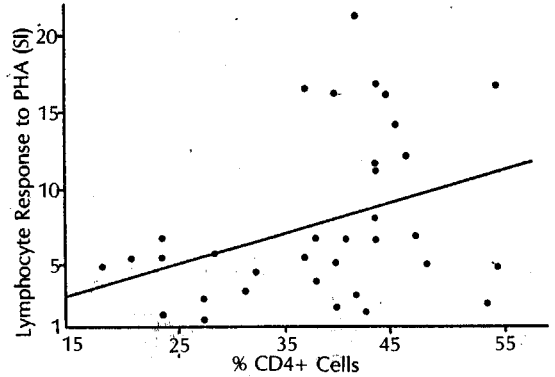
	Invasive cervical carcinoma	
	Early stage (N=18)	Advanced stage (N=16)
Total T cells (%)	62.6±10.4	59.4±11.7
CD4+ cells (%)	37.5±10.0	32.8±10.1
PHA (SI)	7.6± 5.1	8.1± 6.2
Con A (SI)	5.4± 5.6	9.5±11.0
NK cytotoxicity (%)	27.5±17.0	22.8±15.9
ADCC (%)	50.8±15.5	50.6± 5.5
IL-2 productivity (units/ml)	5.2± 4.4	4.9± 3.5

Data are expressed as Mean ± SD.
All differences are not significant.

Fig. 3. Relationship between T helper cell ratio(%) and IL-2 productivity of peripheral blood lymphocytes from patients with invasive cervical carcinoma (linear regression: y=1.13x + 30.77, r-square = 0.20, p<0.01).
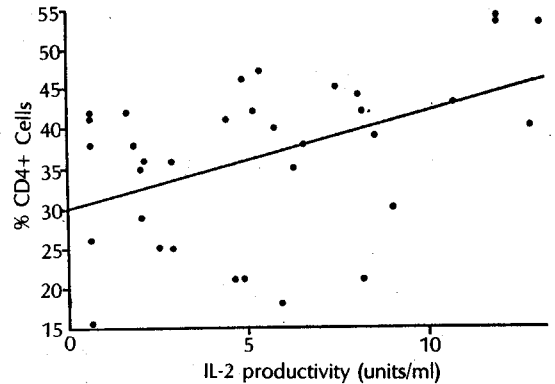
stage. The patients were divided into early stages (stage I and stage II) and advanced stages (stages III and IV). The percentages of T and CD4+ lymphocyte, NK and ADCC activities, and IL-2 activity were slightly lower in advanced stages than in early stages; however, no significant differences were noted.

Regression analysis was performed to examine the relationship between parameters in patients with invasive cervical cancer. The relationship between the percentage of CD4 positive lymphocytes and the PHA response (Fig. 2); the relationship between the percen-

tage of CD4 positive lymphocytes and IL-2 activity (Fig. 3); and the relationship between NK cytotoxic activity and IL-2 activity (Fig. 4) were significant.

DISCUSSION

It has been suggested that immunocompetence of cancer patients is correlated with treatment response and clinical prognosis; if the immune response against the malignant tumor cell is weak, the prognosis may be poor. Therefore, evaluation of immune functions
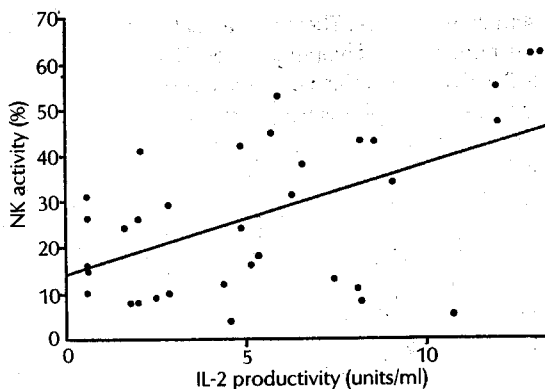
Fig. 4. *Relationship between NK activity and IL-2 productivity of peripheral blood lymphocytes from patients with invasive cervical carcinoma (linear regression: $y=2.27x+15.46$, r-square $=0.28$, $p<0.01$).*

in cancer patients is considered to be of great practical importance. Impairment of general immune reaction in patients with cervical cancer has been demonstrated in several studies (Dutta *et al*. 1977; Rao *et al*. 1977). These studies chiefly involved measurement of percentage and absolute counts of lymphocyte populations and/or their blastogenic responses to various mitogens and have yielded conflicting and inconclusive results. Interactions of various lymphocyte subpopulations and lymphokines have been reported (Gills *et al*. 1979; Granelli-Piperno *et al*. 1982; Seltzer *et al*. 1983; Andrus *et al*. 1984; Rey *et al*. 1984). It seems, therefore, to be important to assess various immune parameters simultaneously. The results of this study demonstrated depressed general immune reactivity in patients with invasive carcinoma of the cevix, as measured by percentage and count of T and B lymphocytes, percentage of T cell subsets, lymphocyte response to PHA and Con A, NK cytotoxicity, ADCC, and IL-2 activity.

In this study, the percentage and count of T lymphocytes were significantly lower in patients with invasive cervical cancer as compared with healthy controls, whereas no significant differences were noted in total lymphocyte and monocyte count, and in the count and percentage of B lymphocytes between cervical cancer patients and normal controls. These findings are in accordance with those of other reports (Rand *et al*. 1977; Levy *et al*. 1978; Utreja *et al*. 1980; Satam *et al*. 1981; Castello *et al*. 1986). Kietlinska (1984) reported decreased T and B lymphocyte count in cervical cancer patients. Several authors observed no differences in T lymphocytes in patients with gynecological cancer compared with

non-malignant controls (Sutherland *et al*. 1971; Hagen *et al*. 1972; Raben *et al*. 1976). These differences among authors may have been due to the use of different test systems or multiple factors which may affect immunological functions in cancer patients. T lymphocytes are known to be concerned with cellmediated immunity which is important in the defense mechanism against tumors. Although there are several reports that are not consistent with ours, this study suggests that the T lymphocyte level is one of the significant indices of cellular immune competence in patients with cervical cancer.

In this study, subsets of T lymphocytes were assayed by using monoclonal antibodies directed against cell surface antigens. The percentage of helper/inducer T (CD4+) lymphocytes in cervical cancer patients was significantly decreased compared to that in controls while a slight increase was noted in the percentage of suppressor/cytotoxic T (CD8+) lymphocytes in patients with cervical carcinoma. This finding is similar to the study of Koech *et al*. (1984) who reported a decrease in the percentage of helper/inducer (OKT4+) T lymphocytes with an increase in that of suppressor/cytotoxic (OKT8+) T lymphocytes in patients with cervical carcinoma. The present study showed a decreased CD4+/CD8+ ratio in cervical cancer patients as compared to controls, which is compatible with the observations reported by Koech *et al*. (1984) and Castello *et al*. (1986).

In accordance with other authors (Jenkins *et al*. 1975; Rafla *et al*. 1978; Koech *et al*. 1984), we found that blastogenic response of lymphocytes from patients with cervical carcinoma to the plant mitogens PHA and Con A was depressed. Daunter *et al*. (1979) observed that the lymphocyte response to PHA in 21 patients with carcinoma of the cervix was decreased and the lymphocyte response to Con A in cancer patients was similar to controls. However, the number of subjects in that study was smaller than in the present one. The plant mitogens PHA and Con A have been shown to stimulate T lymphocytes in humans. This suggests that cell-mediated immunity involving T lymphocytes was impaired in cervical carcinoma patients. The mechanism of impaired T cell function in cancer is not well known. Our data as well as others (Koech *et al*. 1984; Castello *et al*. 1986) suggest any impaired T cell function in cervical cancer may be the result of a decrease in the absolute count of T lymphocytes, an imbalance within the two major T lymphoyte subsets, and/or a lower IL-2 activity.

NK and ADCC activities in cervical cancer patients were lower than those of controls. This finding is in agreement with that of other reports (Seth 1983;

Stewart and Ades 1984; Bose 1985; Yamamoto 1986). There are several hypotheses to explain the reason for low NK cytotoxicity and ADCC in cancer patients. Blocking factors, such as tumor-related immunoglobulins, antigen-antibody complexes, etc., may interfere with this defensive process (Sjogren et al. 1971; Dini and Faiferman 1980; Kristenson et al. 1980; Nair et al. 1980; Karsh et al. 1981; Herberman et al. 1982). The suppressor mononuclear cells may suppress this process in vivo (Broder et al. 1978; Eremin et al. 1981; Uchida et al. 1981). Dawson et al. (1985) suggested that decreased IL-2 production may be one reason for diminishied NK acitvity in squamous head and neck cancer patients. Our data are compatible with this suggestion.

IL-2 has a central augmenting and amplifying role in an immune response to antigen. Rey et al. (1984) suggested that IL-2 production by cancer patient lymphocytes was lowered. The IL-2 production has not been reported in patients with carcinoma of the uterine cervix. The present study demonstrated that peripheral lymphocytes of cervical cancer patients had a lower IL-2 activity than those of controls.

A correlation of the mean percentages of T lymphocyte and T helper/inducer cells, blastogenic response to PHA and Con A, NK cytotoxicity and ADCC, and IL-2 activity with clinical stages revealed that these immune parameters were uniformly depressed and did not vary significantly with the tumor load. Our finding is in agreement with that of Bose et al. (1985) who showed no significant decrease in ADCC in patients with cervical carcinoma with increasing stage of disease. In contrast, other investigators observed depression of T lymphocytes (Utreja et al. 1980) and NK cytotoxicity (Pulay et al. 1982) in cervical cancer patients in correlation with clinical stages. It would appear that immunocompetence declines in advancing tumors, particularly in the presence of widespread malignancy. In the present study, the majority of patients (28) had either stage II or III. No patient had stage IV cervical cancer. Therefore, more cases should be studied to establish this point.

The relationships between the immunologic parameters in patients with invasive cervical cancer were assessed in this study. The percentage of helper/inducer T lymphocytes was associated with the blastogenic response to PHA. Levy et al. (1978) found that low T cell counts were associated with a depressed response to PHA in cervical cancer patients. A relationship between T lymphocyte counts and PHA response was noted in this study. This might be due to the fact that helper T cells are preferentially

stimulated by PHA. There was a relationship between the percentage of helper/inducer T lymphocytes and IL-2 activity and a significant relationship between NK cytotoxicity and IL-2 activity. It was shown in vitro that OKT4- and OKT8-positive cells were able to produce IL-2 (Luger et al. 1982). Recently Dawson et al. (1985) detected a deficiency in the interleukin-2 producing cells in patients with head and neck squamous cell carcinoma. Rey et al. (1984) reported that NK cell activity and the autologous rosette forming (ARF) cell rate are significantly correlated with IL-2 production in solid tumor-bearing cancer patients. It has been shown in vitro that IL-2 causes the proliferation of antigen-specific T helper/inducer cells and also stimulates NK cell cytotoxicity (Morgan et al. 1976; Henney et al. 1981; Kern et al. 1981). It is suggested, therefore, that decreased IL-2 activity could be one mechanism for the impaired T cell and NK cell function in cervical cancer patients.

In summary, the results of the present study indicate a significant defect in an important immune surveillance mechanism in patients with invasive carcinoma of the cervix and suggest that impaired IL-2 activity production may be related to quantitative and qualitative alterations in lymphocyte subpopulations which play a major role in immune surveillance againt cervical cancer.

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