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Characterization of peripheral blood T-cell subpopulation of bladder cancer patients

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Summary. The levels of immune reactivity of peripheral and blood T-lymphocytes were evaluated in 37 bladder cancer patients and 31 age-matched controls. T-lymphocyte subsets were quantified by monoclonal antibodies, and the immune reactivity was measured using stimulation with phytohemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM). Comparing the patients before and after treatment revealed significant changes in the stimulation index of proliferative response to PHA, PWM, in the PWM% (the patient response compared to the control), and in the percent of T8 cells from the total count of blood lymphocytes. Further significant differences were found among the disease stages in the numbers of T3, T4 lymphocytes subpopulations and the total lymphocyte count. A significant interaction was found between the treatment and patient's sex regarding the T4:T8 ratio. Also, a higher prevalence of T4:T8 <1 was found among the patients compared to the controls before and after treatment regardless of the disease stage. This T4:T8 <1 ratio can serve as an indicator of immune competence in bladder transitional cell carcinoma patients.

Key words: Bladder cancer – Peripheral blood T-lymphocytes – Immune response

In recent years there has been a growing interest in the interaction between the immune system activity and certain malignant tumors. It is of interest to study the competence of the immune system in bladder cancer patients, especially since studies have shown bacille Calmette-Guérin (BCG) to have a beneficial effect when administered to these bladder cancer patients [7]. In the present study we evaluated the immune response in patient with bladder transitional cell carcinoma (TCC) by studying the peripheral blood T-lymphocytes subpopulation before and after treatment.

Materials and methods

Thirty-seven patients with transitional-cell carcinoma (TCC) of the bladder were studied before and after treatment. The control group consisted of 31 age-matched patients with benign urological disease. The controls had not suffered previously from acute infectious disease or a malignant disease. Immune-suppressing treatment (such as steroids) was never administered to any of the control patients. The patients were treated in the conventional manner, undergoing surgical removal of the tumor by transurethral resection (TUR-T) and continuing with Thio-Tepa instillation into the bladder (7 patients). Four patients underwent radiation therapy as part of the treatment. The patients were re-evaluated between 5 weeks and 2 years after completing treatment. The first peripheral blood samples were obtained 1 day prior to the TUR-T.

Lymphocyte isolation and mitogenic stimulation test

Lymphocytes were isolated from heparinized peripheral blood by centrifugation over Ficoll-Isopaque gradient (Pharmacia). Lymphocytes at a concentration of 1.5×10^6 cells/ml in RPMI-1640 medium (containing 5% human AB plasma; penicillin, 100 U/ml; and streptomycin, 100 µg/ml) were cultured in round-bottom microculture plates (Grainer, FRG). Phytohemagglutinin (PHA, Wellcome Research Labs, England), concanavalin A (ConA, Bio-Yeda, Israel), and pokeweed mitogen (PWM, Gibco) were added to the triplicate cultures. Dose response curves for mitogenic stimulation of human peripheral blood lymphocytes had been established previously in our laboratory [3], and optimal concentrations were selected for this study. Two concentrations were used for each mitogen: PHA 2 µg/ml and 4 µg/ml, ConA 8 µg/ml and 12 µg/ml, and PWM 1:20 and 1:50. The microcultures were incubated in an atmosphere of CO_2 5% and air 95% at 37°C for 72 h. Each well was then pulsed with 1 µCi of thymidine (^3H) and incubated for further 4 h. The cells were harvested (Automatic harvester, Dynatech) onto glass-fiber filter paper and the incorporation of thymidine was measured as counts per minute (cpm) with a liquid scintillation counter (Packard). The mitogenic responses were calculated as mean cpm for triplicate cultures of the maximal response, of the two mitogen concentration tested. Two calculations were made:

$$1.\ \frac{\text{cpm of patients} \times 100}{\text{cpm of controls}} = \%$$

The results of the control subjects were taken from a test done concomitantly (on the same day using the same reagents) with the patient lymphocytes.

Table 1. The distribution of patients according to the disease stage

Stage	Before treatment		After treatment	
	No. of patients	Percent	No. of patients	Percent
0	1	2.7	1	2.7
A	22	59	20	54
B, C, D	14	14	16	43

2. $\frac{\text{cpm (mitogen + lymphocytes)}}{\text{cpm (lymphocytes without mitogen)}}$
= stimulation index (SI)

The highest stimulation index obtained from the two concentrations of the mitogen was used in the study.

T-cell phenotypic analysis

Mononuclear cell suspensions at a concentration of 1×10^6, cells/ml were interacted with leu3 (T4) and leu2 (T8) and leu4 (T3) monoclonal antibodies (Becton Dickinson) and incubated on ice for 30 min in the dark. The cells were washed three times with phosphate buffered saline (PBS), and incubated with 25 µl goat anti-mouse fluoresceinated (FITC) IgG (Bio-Yeda, Israel) for 30 min on ice in the dark. They were then rewashed and resuspended in PBS and percentage of stained cells was determined with the fluorescence activated cell sorter (FACS II). The absolute number per cubic millimeter of T4 and T8 subsets was calculated using the number of lymphocytes obtained from the differential count of peripheral blood white blood cells. The mean T4:T8 ratio of each group was calculated from the individual ratio obtained for each patient.

Results

The subjects were 30 males and 7 female patients. The average age was 68 years (+9.5, range 42–84). These parameters had not changed significantly at the termination of the study. The control group consisted of 31 healthy men with an average age of 70.3 years (±9.5 range = 39–84).

The distribution of patients according to the disease stage is given in Table 1. Tables 2 and 3 describe the parameters used in this study, as measured before and after treatment. Paired comparisons were made using the

Table 2. The variables of response to mitogenic stimulation of treated patients before and after treatment

	Before					After				
Stage	O	A		B, C, D		O	A		B, C, D	
Variable	M	M	SD	M	SD	M	M	SD	M	SD
SI PHA	116.0	181.5	111.2	146.3	104.8	45.0	85.2	82.7	135.5	160.3
SI ConA	40.0	58.9	40.9	31.2	27.9	19.0	38.9	27.9	37.4	42.1
SI PWM	46.0	50.5	41.1	38.1	49.6	25.0	17.3	17.8	26.5	29.3
PHA %	18.0	119.8	108.4	101.5	84.5	125.0	101.9	83.2	60.0	54.2
ConA %	12.0	93.5	47.2	78.0	70.3	82.0	88.5	69.1	101.5	204.1
PWM %	34.0	122.5	153.7	113.1	106.5	25.0	70.2	69.9	110.5	258.9

M = mean; SD = standard deviation

Table 3. The variables of monoclonal antibody response of treated patients before and after treatment

	Before					After				
Stage	O	A		B, C, D		O	A		B, C, D	
Variable	M	M	SD	M	SD	M	M	SD	M	SD
T3 %	54.0	64.3	147.0	59.9	15.3	37.0	64.9	14.5	60.3	16.7
T4 %	24.0	44.2	17.4	40.0	19.5	16.0	45.5	17.8	39.5	16.0
T8 %	35.0	26.9	14.7	26.7	11.7	39.0	32.2	19.6	35.0	13.1
LYM no.	1,300.0	2,109.5	459.2	1,669.2	347.3	2,200.0	2,200.0	671.2	1,466.8	735.3
T3 no.	700.0	1,366.6	465.1	992.8	324.9	814.0	1,368.3	505.0	879.3	324.3
T4 no.	310.0	928.8	478.1	669.5	349.5	352.0	943.6	430.8	604.6	302.5
T8 no.	450.0	586.8	440.7	436.1	172.1	858.0	646.3	390.9	531.0	253.8
T4:T8	0.7	2.2	1.7	1.9	1.5	0.4	2.3	1.9	1.3	0.97

M = mean; SD = standard deviation

Table 4. Variables in the control group

No.	Variable	Mean	Standard deviation
1	Age (year)	70.3	9.5
2	SI PHA	140.9	153.7
3	SI ConA	89.6	111.6
4	SI PWM	53.6	54.9
5	T3 %	58.9	13.9
6	T4 %	45.3	15.6
7	T8 %	27.2	19.6
8	Lymphocytes (no.)	2,073.9	543.7
9	T3 (no.)	1,217.0	428.8
10	T4 (no.)	923.8	362.5
11	T8 (no.)	589.3	388.3
12	T4:T8	2.3	1.3

T-test. Table 4 describes the mean and standard deviation of the control group parameters.

A two-way analysis of variance (ANOVA) (disease stage, testing time) was performed on the data, with testing time being the within-subject variable. The analysis disclosed a significant main effect of the disease stage on T3 ($P<0.001$) T4 ($P<0.001$) and SI ConA and total counts of lymphocytes ($P<0.04$). Testing time had a significant effect on the parameter SIPWM ($P<0.01$). The interaction within disease stage and testing time did not reach the 0.05 significance level. Further ANOVAS did not reveal that the patient's sex had any effect except for the variable T4:T8 where a significant interaction of the subject's sex × testing time was detected ($P<0.001$).

Due to the small number of female patients in this study, their data were excluded, and the remaining data (only the male patients) was reanalyzed. There were significant effects for testing time for the variables SIPWM and %PWM (both at $P<0.01$). The effect was not significant for the %T8 variable and was only marginal for the SIPHA variable ($P<0.06$).

The analysis also included comparisons between the patients and the control group. Before treatment, the patients differed from the controls on the SIConA variable ($P<0.02$). After treatment, both SIConA and SIPWM differed significantly between the two groups ($P<0.01$ and $P<0.005$), respectively. Excluding the female patients' data revealed significant differences between the two groups before treatment on SIConA ($P<0.02$) and T4:T8 ($P<0.04$). After treatment, the groups (excluding the female data) differed significantly on SICONA ($P<0.01$) and SIPWM ($P<0.005$). It should be noted that after treatment the significant difference between the groups on the T4:T8 variable was diminished.

The ratio T4:T8 was divided into two values: greater than one or smaller than one. A Fisher exact test comparison the patients before treatment to the controls had a T4:T8 ratio of less than 1. After treatment, this difference persisted, including the female patients data in this analysis did not change the differences between the patient group and the controls.

Discussion

The pioneering study of Bubenik in 1970 [2] demonstrating the antigenic effect of bladder TCC led to increasing interest in the relationship between urological tumors and immune system activity. The cytotoxic effect of peripheral blood lymphocytes on TCC was studied by O'Toole, who also investigated the different stages of the disease and the various treatment means used [10, 11]. In the past, the absolute number of peripheral blood lymphocytes served as a predictive parameter. Brossman [1] combined in vivo measures such as a skin test using dinitrochlorobenzen (DNCB) with in vitro tests like PHA-dependent cytotoxicity and their improvement after treatment. His findings were suported by Nishio [8]. Following the introduction of monoclonal antibodies into the study of T-lymphocytes [12], the relevance of lymphocytes subsets to immune regulation was highlighted. Using monoclonal antibodies and measuring various subsets of T-lymphocytes, Harris [4] failed to predict the cource of the disease. A different approach was used by Shaw [13], who evaluated T-lymphocytes subsets by monoclonal antibodies in urological cancer patients. Patients with bladder cancer demonstrated an increased suppressor cell activity and a decreased T4:T8 ratio. These findings were prevalent in the advanced disease stage.

In the present study, the characteristics of peripheral blood T-lymphocytes of TCC patients were studied and compared to a control group. The effects of treatment on these characteristics were also studied. The lymphocyte response to mitogenic stimulation was observed using PHA, ConA, and PWM. In addition, the distribution and relationship of the T-cell various subsets were measured. Treatment had a significant effect on the parameters SIPHA, SIPWM, %PWM, and %T8. The mitogenic response decreased after treatment and the %T8 was elevated. These may reflect the reactivity of the immune system in order to restore a normal steady state [6]. The changes in T3, T4, Si ConA and lymphocytes count were related to the disease stage with no treatment effect. We failed to replicate Shaw's finding [13] and did not find any difference among the disease stages in the T8 parameter. In our study patient sex and treatment interacted with regard to the T4:T8 ratio. This may be a partial explanation of the favorable prognosis of bladder TCC in females [5]. The female patients accounted for only 20% of our group, and their exclusion from the analysis revealed a significant difference between the patients and controls on the SI PWM and %PWM variables. Comparing the patients to the controls revealed significant differences on the SIConA (before) and SIConA (after). Comparing only the men in both groups also indicated a significant difference in the T4:T8 ratio before treatment. This difference disappeared after treatment. This T4:T8 ratio reflects the relationship between the helper T-cells (T4) and the suppressor T-cells (T8). Treatment affected this ratio, whether it was greater or smaller than one, with no relationship to the disease stage. The groups also differed regarding the T4:T8 ratio, more patients having a ratio T4:T8 of less than one than the controls. This distinctive immunological pattern, which differentiates

the bladder TCC patients from the controls, was reported by Shaw [13]. However, without reference to whether the ratio was greater or smaller than 1, he only demonstrated this change in invasive tumors.

The activity of the T-lymphocytes subsets is dependent on various factors, such as stress, operation, treatment, systemic chemotherapy, and radiation [9]. We aimed at comparing the patient population to a healthy control group without the effect of surgery, etc. Therefore, the effect of those factors was not evaluated in our control group. Because of this factor, stimulation tests using PHA, ConA, and PWM were less reliable than the monoclonal antibodies testing.

The consistent finding of an altered T-lymphocyte distribution, as reflected by a T4:T8 ratio of less than 1, distinguished the patient group from the control group. We believe that the use of this parameter may prove to be valuable and that it represents an immunological pattern that differs patients from control, subjects even in the disease-free period.

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