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EVALUATION OF LYMPHOCYTE SUBPOPULATIONS IN PATIENTS WITH BLADDER CANCER: A STUDY WITH MONOCLONAL ANTIBODIES

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Peripheral blood lymphocyte subpopulations of 47 patients with bladder cancer were analyzed using monoclonal antibodies (OKT3, OKT4, OKT8 and OKIa7). Thirty five patients were untreated, and 12 were treated, disease-free patients. In untreated patients, the number of total lymphocytes and the percentage of $OKT3^+$ cells decreased. Among the $OKT3^+$ T cell population, the percentage of $OKT4^+$ cells tended to decrease significantly, whereas the percentage of $OKT8^+$ cells rather increased, resulting in the decrease of the $OKT4^+/OKT8^+$ ratio. These tendencies became more evident as the stage advanced. In treated, disease-free patients, no such characteristic changes were observed and the lymphocyte subpopulation apparently fell into the normal range. Furthermore, the retrospective study clearly indicated that the patients with a lower $OKT4^+/OKT8^+$ ratio showed a higher recurrence rate within a year.

Analysis of peripheral blood lymphocyte subpopulations thus appears to be useful in the follow-up of bladder cancer patients, especially those with superficial tumors.

Key words: Lymphocyte subpopulations, Bladder cancer patients

INTRODUCTION

A variety of immunological parameters, including *in vitro* blastoid response to PHA or ConA[1], *in vitro* nonspecific cytotoxic activity[2] and delayed hypersensitivity skin reactions with recall antigens[3], have been reported to be impaired to various degrees in bladder cancer patients. Such immunological responses are mediated and/or regulated by a number of distinct sets of immunocompetent cells. Since these assays are technically difficult to perform, direct measurement of the numbers of each set of immunocompetent cells would provide a more stable and precise way to detect the basic immunological status of the patients.

Recently, a series of monoclonal antibodies that react with different lymphocyte subpopulations have been developed, and they would be valuable in precisely quantitating the lymphocyte subpopulations. Using such monoclonal antibodies, lymphocyte subpopulations have been systemically analyzed in various diseases, such as leukemia[4], autoimmune diseases[5], viral infections[6] and some solid cancers[7~11], and the analysis of lymphocyte subpopulations was found to be quite useful in monitoring the immunological status of the patients, and, in some occasions, in helping diagnosis as well as predicting the prognosis of diseases. For instance, abrupt reversion of $OKT4^+/OKT8^+$ ratio in immunosuppressed patients was suggested to be highly associated with viral infections such as cytomegalovirus (CMV) mononucleosis, whose exact diagnosis is not easy and time-consuming[12].

In the present study, we systemically evaluated the peripheral blood lymphocyte subpopulations in bladder cancer patients to ascertain the possible relationship

Table 1. Characteristics of controls and patients.

	Controls	Bladder Cancer Patients	
		Untreated.	Treated.
Total number	20	35	12
Mean age (range)	62(44-70)	64(41-84)	65(52-80)
Sex M F	3 1	4 :1	3 1
TNM classification			
Low stage		23	
(Tis N0 M0, T1 N0 M0, T2 N0 M0)			
High stage		12	
(T3 Nx M1, T4 Nx M1)			

between the patterns of lymphocyte subpopulations and clinical states in terms of cancer stage, operative resection and the frequency of recurrence.

MATERIALS AND METHODS

Patients

Forty-seven patients with transitional cell carcinoma of the bladder were studied. They were 35 untreated patients including 25 patients with recurrent disease; and 12 treated, disease-free patients. The patients in group 2 continued to be free of tumor at least one year after transurethral resection (TUR) of the tumors. The stage of disease was determined by routine investigative procedures and operative findings according to TNM staging system. The control group consisted of healthy volunteers with a similar age and sex distribution as the patient population. Table 1 shows their detailed characteristics. None of the patients had received either chemotherapy or radiation therapy before. In addition, all patients and controls entered in this study had no signs or symptoms of infection, and had not used medications which could effect the lymphocyte subpopulation analysis.

Blood samples were collected into containers with heparin as an anticoagulant, at the same time of day (11:00 a.m.-12:00 a.m.) to avoid circadian variation of lymphocyte subpopulations[13].

Monoclonal Antibodies (MoAbs)

Monoclonal antibodies used in the study were OKT series (Ortho Pharmaceutical Co., Raritan, NJ) including OKT3, OKT4, OKT8 and OKIa1. OKT3 is a mature T cell marker, whereas OKT4 and OKT8 identify helper/inducer and suppressor/cytotoxic T cell subsets, respectively. OKIa1 identifies B cells and activated T cells.

Immunofluorescence Staining and Analysis

In direct immunofluorescence staining, 100 μl of samples were incubated with 10 μl of FITC-conjugated MoAbs (OKT3, OKT4 and OKT8) at 4°C for 30 min. In indirect immunofluorescence staining, 100 μl of samples were incubated with 5 μl of MoAb (OKIa1) at 4°C for 30 min, then FITC-conjugated goat anti-mouse IgG F $(ab')_2$ was added and they were incubated at 4°C for another 30 min.

After each incubation, 2 ml of a lysing reagent (8.29 g NH_4Cl, 37 mg ethylenediaminetetraacetic acid, and 1 g $KHCO_3$ per liter, pH 7.4) were added and erythrocytes were lysed at room temperature for 30 min. Labeled lymphocytes were washed three times in phosphate buffer solution (PBS) and resuspended in 1 ml of PBS.

All samples were then analyzed for immunofluorescence in a flow cytometry (Ortho Spectrum III, Ortho Diagnostic System, Westwood, MA) as previously described[14]. Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90° light scatter profile. Ten thousand cells were analyzed for each antibody, and

the number of fluorescinated cells were expressed as the percentage of the total lymphocytes.

Lymphocyte Count

A Coulter counter was used to determine the white blood cell count in all blood samples, and lymphocyte counts were calculated from the percentage of lymphocytes estimated by differential analysis on smears.

Statistics

The results were statistically analyzed for significance by Student's *t*-test.

RESULTS

Lymphocyte Subpopulations in Untreated Bladder Cancer Patients

Table 2 shows the number of lymphocytes and the percentage of each lymphocyte subpopulation in untreated bladder cancer patients. The number of total lymphocytes and the percentage of $OKT3^+$ cells in cancer patients were significantly lower than those in normal controls. Among the $OKT3^+$ T cell population, the percentage of $OKT4^+$ cells tended to significantly decrease, whereas the percentage of $OKT8^+$

Table 2. Lymphocyte subpopulations in patients with bladder cancer.

	Lymphocytes (cells/μl)	$OKT3^+$ (%)	$OKT4^+$ (%)	$OKT8^+$ (%)	$OKT4^+/OKT8^+$ ratio	$OKIa1^+$ (%)
A-Controls (n=20)	2405±343*	70.5±8.8	48.4±6.4	24.0±3.4	2.1±0.3	16.9±4.1
B-Low stage (n=23)	1983±324	65.2±10.7	36.9±4.9	31.7±3.7	1.25±0.42	17.9±6.3
C-High stage (n=12)	1193±374	57.3±7.0	29.3±6.1	33.8±2.8	0.86±0.27	18.7±9.5
Student's t-test						
A vs B	P<0.05	NS†	P<0.01	P<0.05	P<0.01	NS
A vs C	P<0.01	P<0.05	P<0.01	P<0.01	P<0.01	NS
B vs C	P<0.05	NS	P<0.01	NS	P<0.05	NS

* Mean ± SD

† Not Significant

Table 3. Lymphocyte subpopulations in untreated patients and treated, disease-free patients.

	Lymphocytes (cells/μl)	$OKT3^+$ (%)	$OKT4^+$ (%)	$OKT8^+$ (%)	$OKT4^+/OKT8^+$ ratio	$OKIa1^+$ (%)
A-Controls (n=20)	2405±343*	70.5±8.8	48.4±6.4	24.0±3.4	2.1±0.3	16.9±4.1
B-Untreated pts (n=35)	1672±567	62.1±10.0	33.9±5.8	32.6±3.3	1.1±0.4	18.2±7.6
C-Treated pts (n=12)	2640±353	61.9±10.1	42.3±5.0	24.5±3.6	1.9±0.3	19.7±8.0
Student's t-test						
A vs B	P<0.05	NS†	P<0.01	P<0.05	P<0.01	NS
A vs C	NS	NS	NS	NS	NS	NS
B vs C	P<0.05	NS	P<0.05	P<0.05	P<0.05	NS

* Mean ± SD

† Not Significant

cells to increase, resulting in the decrease of the OKT4+/OKT8+ ratio. Such tendencies were more evident as the disease stage advanced. In contrast, the ratio of OKIa1+ cells, which primarily represented the B cells, remained quite constant as normal controls irrespective of disease stage.

Lymphocyte Subpopulations in Treated, Disease-free Patients

The effect of surgical resection of primary tumors on lymphocyte subpopulations was then examined. As shown in Table 2, untreated tumor-bearing patients showed characteristic changes of lymphocyte subpopulations as above, namely the decrease in OKT4+ cells and the increase in OKT8+ cells. In treated, disease-free patients, on the other hand, both OKT4+ cells and OKT8+ cells showed a clear tendency to normalize (Table 3). OKT4+/OKT8+ ratio was thus almost equivalent to normal controls in this group. The OKIa1+ population again remained constant in disease-free patients.

Correlation between the OKT4+/OKT8+ Ratio and the Recurrence Rate

Since our findings suggested that the OKT4+/OKT8+ ratio was the most sensitive parameter to reflect the stage of the disease as well as the effect of treatment, the relationship between OKT4+/OKT8+ ratio and the frequency of recurrence was next examined. The patients with low stage bladder cancer and those who had been treated to be disease-free were divided into five groups depending upon the frequency of recurrence in the last one year from the analysis study, and the OKT4+/OKT8+ ratio was examined.

As shown in Fig. 1, the patients without recurrence retained a basically normal range of OKT4+/OKT8+ ratio, whereas those with recurrence showed progressive decrease in the OKT4+/OKT8+ ratio as the frequency of recurrence increased.

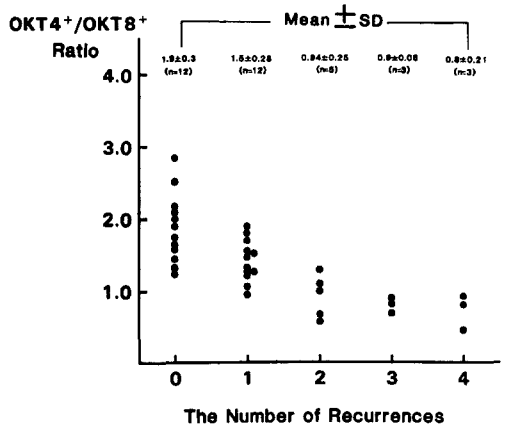

Fig. 1. Correlation between the OKT4+/OKT8+ ratio and the number of recurrences in the last one year.

DISCUSSION

Analysis of the lymphocyte subpopulation in bladder cancer patients have already been reported using spontaneous sheep red blood cell (SRBC) rossette formation (T cell) and surface immunoglobulin detection (B cell). Catalona and associates reported that the T lymphocyte level was significantly reduced and appeared to correlate inversely with tumor stage in bladder cancer patients[15]. Shimatani reported that there was a tendency of increasing percentage of Tγ cells bearing receptors for the Fc portion of IgG among T cells in patients with a higher grade and stage of bladder cancer[16].

In the present study, untreated bladder cancer patients showed a decrease in the OKT4+ subset and an increase in the OKT8+ subset, resulting in a significant decrease in the OKT4+/OKT8+ ratio as the stage advanced. Similar results of stage-related decrease in OKT4+/OKT8+ ratio have been reported in other soild cancers, such as malignant melanoma[7,8] breast cancer[9], prostatic cancer[10] and renal cell carcinoma[11]. This numerical alteration in lymphocyte subpopulation may account for the immunosuppression demonstrated by other immunological examinations in bladder cancer patients[17].

At present, the exact mechanism of the characteristic changes of the OKT4+/OKT8+ ratio in the peripheral blood in cancer patients is not clear. Our present results indicate that the OKT4+/OKT8+ ratio in tumor-free patients reverted to normal compared with that in tumor-bearing patients, whereas it progressed to

reduce as the tumor advanced. These results thus suggest that the change in the $OKT4^+/OKT8^+$ ratio was primarily associated with the presence of tumor masses, whatever the mechanism is. To confirm this point, a sequential study of lymphocyte subpopulation in each patient would be needed.

One of the most serious problems encountered in cases of superficial bladder cancer is the high incidence of recurrence after initial treatment by trasurethral resection. Of these patients, 50 to 70 percent can be expected to have recurrence after complete resection of all visible lesions[18]. Thus early detection of recurrent bladder cancer after initial treatment is still a major concern in the follow-up of this disease. Several parameters have been proposed to help us to determine whether tumors are likely to recur[19]. Among them, measurement of cellular-immunity seems to have a predictive value[20]. In our study, bladder cancer patients with higher recurrence rates showd a lower $OKT4^+/OKT8^+$ ratio even if tumors were superficial. A similar tendency was also reported in malignant melanoma patients. Maria Grazin Bernengo et al. reported that some patients with malignant melanoma who presented a constantly low $OKT4^+/OKT8^+$ ratio developed metastasis during the follow-up[8]. Although it is not clear whether this reduction in $OKT4^+/OKT8^+$ ratio is a causative factor or a consequence of the disease, these results suggest that monitoring of the $OKT4^+/OKT8^+$ ratio might be very useful for predicting the recurrence of this disease.

In conclusion, we have shown that untreated bladder cancer patients have a reduced $OKT4^+/OKT8^+$ ratio and this reduced $OKT4^+/OKT8^+$ ratio was inversely correlated with the recurrence rate.

REFERENCES

1) Catalona WJ, Tarpley JL, Chretien PB and Castle JR: Lymphocyte stimulation in urologic cancer patients. J Urol **112**: 373, 1974
2) Morales A and Ottenhof PC: Clinical application of a whole blood assay for human natural killer (NK) activity. Cancer **52**: 667, 1983
3) Olsson CA, Rao CN, Menzoian JO and Byrd WE: Immunologic unreactivity in bladder cancer patients. J Urol **107**: 607, 1972
4) Hattori T, Uchiyama T, Toibana T, Takatsuki K and Uchino H: Surface phenotype of Japanese adult T-cell leukemia cells characterized by monoclonal antibodies. Blood **58**: 645, 1981
5) Veys EM, Hermanns P, Goldstein G, Kung P, Schindler J and Van Wauwe J: Determination of T lymphocyte subpopulations by monoclonal antibodies in rheumatoid arthrities, influence of immunomodulating agents. Int J Immunopharmacol **3**: 313, 1981
6) De Waele M, Thielemans C and Van Camp BKG: Characterization of immunoregulatory T cells in EBV-induced mononucleosis by monoclonal antibodies. N Engl J Med **304**: 460, 1981
7) Karavodin LM, Giuliano AE and Golub SH: T lymphocyte subsets in patients with malignant melanoma. Cancer Immunol Immunother **11**: 251, 1981
8) Bernengo MG, Lisa F, Puiatti P, Meregalli M, Berruto G and Zina G: T-cell subsets in melanoma patients evaluated by anti-T-cell monoclonal antibodies. Thymus **5**: 223, 1983
9) McCluskey DR, Roy AD, Abram WP and Martin WMC: T lymphocyte subsets in the peripheral blood of patients with benign and malignant breast disease. Br J Cancer **47**: 307, 1983
10) Rubenstein M, Shaww MW, McKiel CF, Stobnicki M, Ablin RJ and Guinan PD: The effect of hormone therapy on peripheral blood leukocytic subset distribution in stage D prostatic cancer patients. Urol Res **13**: 67, 1985
11) Ritchie AWS, James K, Micklem HS and Chisholm GD: Lymphocyte subsets in renal carcinoma—a sequential study using monoclonal antibodies. Br J Urol **56**: 140, 1984
12) Carney WP, Rubin RH, Hoffman RA, Hansen WP, Healey K and Hirsch MS: Analysis of T lymphocyte subsets in cytomegalovirus mononucleosis. J Immunol **126**: 2114, 1981
13) Ritchie AWS, Oswald I, Micklem HS, Boyd JE, Elton RA, Jazwinska E and James K: Circadian variation of lymphocyte subpopulations: a study with monoclonal antibodies. Br Med J **286**: 1773, 1983
14) Hoffman RA, Kung PC, Hansen WP and Goldstein G: Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. Proc Natl Acad Sci **77**:

4914, 1980
15) Catalona WJ, Potvin C and Chretien PB: T lymphocytes in bladder and prostatic cancer patients. J Urol **112**: 378, 1974
16) Shimatani N: Studies on T cell bearing Fc receptors for IgG of peripheral lymphocytes in patients with bladder carcinoma. Acta Urol Jpn **27**: 637, 1981
17) Catalona WJ: Commentary on the immunobiology of bladder cancer. J Urol **118**: 2, 1977
18) Greene LF, Hanash KA and Farrow GM: Benign papilloma or papillary carcinoma of the bladder? J Urol **110**: 205, 1973
19) Dalesio O, Schulman CC, Sylvester R, Pauw MD, Robinson M, Demis L, Smith P, Viggiano G and Members of the European Organization for Research on Treatment of Cancer, Genitourinary Tract Cancer Cooperative Group: Prognostic factors in superficial bladder tumors. A study of the European Organization for research on treatment of cancer: Genitourinary Tract Cancer Cooperative Group. J Urol **129**: 730, 1983
20) O'Boyle PJ, Cooper EH and Williams RE: Evaluation of immunologic reactivity in bladder cancer. Br J Urol **46**: 303, 1974

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和文抄録

膀胱癌患者におけるリンパ球サブポピュレーションの検討
—モノクローナル抗体による解析—

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湊長博

膀胱癌患者47人の末梢血リンパ球サブポピュレーションを，モノクローナル抗体（OKT3, OKT4, OKT8, OKIa1）を用いて，フローサイトメトリーにて解析した．患者は，(i) 未治療患者35人，(ii) 既治療患者12人の2群に分け，健康人20人をcontrolとして検討した．未治療患者では，controlに比し，リンパ球数およびOKT3陽性細胞の割合の低下が認められた．さらに，OKT4陽性細胞の減少，OKT8陽性細胞の増加により，OKT4/OKT8比の低下が認められた．この傾向は，high stage膀胱癌患者において，より著明であった．一方，既治療患者では，controlに比し，著明なサブポピュレーションの変化は認められなかった．また，low stage膀胱癌患者において再発率（最近1年間における再発回数）とOKT4/OKT8比との関係をみると，再発率の高い患者では，OKT4/OKT8比がより低下する傾向がみられた．

以上より，末梢血リンパ球サブポピュレーションの解析は，膀胱癌患者—特に，low stage膀胱癌患者—のfollow-upに有用であろうと考えられた．