RESTORATION BY LEVAMISOLE OF E-ROSETTE FORMATION AND ITS ABROGATION BY AUTOCHTHONOUS SERUM FROM PATIENTS WITH BLADDER CANCER

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Incubation *in vitro* of lymphocytes from bladder cancer patients with levamisole (40 μg/ml) resulted in a rise of the number of E-rosette forming cells from 39.2 ± 11.8% to 57.5 ± 11.3% ($p < 0.005$). The same effect was observed when levamisole was administrated 150 mg/day for 3 days to the patients. The stimulatory effect of levamisole was abrogated when the lymphocytes were first incubated with levamisole and afterwards with 50% autochthonous serum from the patient. With more diluted serum concentrations (from 0.5% to 1%), the response decreased, and the response was not observed with allogeneic serum. When lymphocytes from healthy donors were incubated with 50% serum from bladder cancer patients, there were no significant changes in numbers of E-rosette forming cells. It was presumed that the suppression of E-rosette forming cells from the patients with bladder cancer was caused by blocking the receptor sites for sheep red blood cells by autochthonous serum components and that levamisole eliminated such substances from the cryptic sites on the surface of the lymphocytes.

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LEVAMISOLE (1-2,3,5,6-tetrahydro-6-phenylimidasole [2,1-b] thiazole hydrochloride) is a widely used anthelmintic drug. About 7 years ago, Renoux and Renoux[12] demonstrated that this drug stimulated the immune system of mice. Since then, many investigators have been trying to study how the drug enhances immune responses and whether it will do so in human cancer patients with defective immunity. Recently, it became obvious that administration of the drug increased E-rosette forming cells in patients with various malignancies and also that it increased the number of such cells when lymphocytes from patients were treated with the drug *in vitro*.[5,14] We studied the mechanism of the suppression of E-rosette forming cells from the patients with bladder cancer and its abrogation by the drug.

PATIENTS AND METHODS

In vitro studies were performed on lymphocytes from 21 patients with bladder cancer of stage 3 and 4 under the TNM system proposed by the International Union Against Cancer, and 8 normal controls. All patients were preoperative and none of the patients had received radiation therapy or chemotherapy. The mean age of patients was 61 years and healthy controls was 46 years. Method for lymphocytes separation and purification was according to that of Tebbi[13] as modified by Ammpo and Kumagaya[2] with carbonyl iron for depletion of phagocytic cells and with Ficoll-Hypaque gradient for lymphocyte isolation. E-rosette assay for T lymphocytes was performed according to the method of Wybran and Fudenberg,[15] and EAC-rosette assay for B lymphocytes according to the method of Raban *et al.*[10] To 1.0 ml of lymphocytes suspension in RPMI-1640 (pH 7.4) (2×10^6/ml) which contained 10% fetal calf serum (FCS) was added levamisole with the final concentration of 40 μg/ml and incubated at 37 C for 40 minutes. The mixture was centrifuged

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TABLE 1. Effect of Levamisole of E-Rosette Forming Cells *in Vitro*

Lymphocytes donors	E-rosette forming cells (%)	
	Before levamisole treatment	After levamisole treatment
Controls (8)	69.6 ± 7.5*	69.3 ± 8.5
Patients (21)	39.2 ± 11.8	57.5 ± 11.3

* Mean ± SD.
$p < 0.005$ for before levamisole treatment vs after levamisole treatment in patients with bladder cancer.
Number of cases examined in parentheses.

TABLE 2. Effect of Levamisole of EAC-Rosette Forming Cells *in Vitro*

Lymphocytes donors	EAC-rosette forming cells (%)	
	Before levamisole treatment	After levamisole treatment
Controls (8)	21.7 ± 7.9*	19.7 ± 7.0
Patients (21)	26.0 ± 6.6	23.2 ± 5.6

*Mean ± SD.
Numbers of cases examined in parentheses.

at 200 × *g* and the lymphocytes sediment was washed with the RPMI-1640 medium three times. A part of the washed lymphocytes was resuspended in FCS and used for rosette assays. The remaining washed lymphocytes were resuspended in 1.0 ml of 50% autochthonous serum and reincubated at 37 C for 40 minutes. In some cases, the washed lymphocytes were resuspended in more diluted autochthonous serum by RPMI-1640 medium (from 1% to 0.5%) and also in 50% decomplemented allogeneic serum. In some healthy controls, the lymphocytes were suspended in 50% decomplemented cancer serum and incubated at 37 C for 40 minutes. The incubated solution was centrifuged at 200 × *g* and the sediment was washed three times with RPMI-1640 medium and resuspended in FCS. E-rosette formation was counted. In every step, the lymphocytes viability was greater than 95% by trypan blue exclusion. In 5 bladder cancer patients, levamisole was administered 150 mg/day for 3 days, and E and EAC-rosette formation were examined before and after the administration.

RESULTS

The effects of levamisole on E-rosette forming cells from patients with bladder cancer and normal controls are shown in Table 1. The drug raised significantly the number of E-rosette forming cells from patients with bladder cancer from 39.2 ± 11.8% to 57.5 ± 11.3% ($p < 0.005$). No such effects were observed on the lymphocytes from controls. This drug has no effect on EAC-rosette forming cells in both groups as shown in Table 2. The effects of levamisole on E-rosette forming cells from bladder cancer patients were completely abolished after reincubation with 50% autochthonous serum as shown in Table 3. This E-rosette suppressive effect of autochthonous serum decreased by the dilution of the serum as shown in Table 4. The E-rosette suppressive effect was not observed with allogeneic serum as shown in Table 5. When lymphocytes from healthy donors were incubated with 50% serum from bladder cancer patients, there were no significant changes in number of E-rosette forming cells as shown in Table 6. When levamisole was administered 150 mg/day for 3 days to bladder cancer patients, E-rosette forming cells significantly increased compared with that before the administration whether EAC-rosette forming cells were not significantly changed as shown in Table 7, whereas the absolute lymphocytes counts were unchanged. These results indicate that the serum from bladder cancer patients contains the components which inhibit

TABLE 3. Abrogation of Restorative Effect of Levamisole on E-Rosette Forming Cells by 50% Autochthonous Serum

Lymphocytes donors	E-rosette forming cells (%)		
	Before levamisole treatment	After levamisole treatment	After treatment with autochthonous serum (50%)
Controls (4)	65.8 ± 6.3*	64.2 ± 5.0	58.5 ± 5.7
Patients (8)	31.8 ± 11.0*[1]	50.8 ± 9.6*[2]	30.0 ± 11.6*[3]

* Mean ± SD.
$p < 0.01$ for *[2] vs *[3] and NS for *[1] vs. *[3].
Numbers of cases examined in parentheses.

E-rosette formation and that levamisole eliminates such components.

DISCUSSION

It is now acknowledged that cellular immune system is impaired at varying severity at different stages of malignancy.[3,8,11] This is also the case with urologic cancer patients.[3,8] In patients with Hodgkin's disease or with various cancers, levamisole has been shown to restore the number of lymphocytes which are involved in *in vitro* E-rosette formation.[5,7,11,14] The mechanism accounting for this phenomenon is unexplained. Recently, Wybran *et al.*[16] reported that levamisole significantly increased the response to PHA of T cell rich human lymphocytes. Kaplan *et al.*[6] reported that the number of T cells in the peripheral blood from patients with Hodgkin's disease, when detected by an anti-T-antiserum was normal, whereas that of T cells was low when detected by E-rosette formation. These results suggested that levamisole had effects on lymphocytes metabolites and function. Our results suggested that the T cells from patients with bladder cancer were functionally abnormal, probably due to blocking their sheep red blood cell receptors by the serum components, and that levamisole may restore the ability of T cells to form rosette by eliminating the serum components (blocking factor?) from the receptors. The suppression of E-rosette formation by serum factors was found to be concentration dependent and these factors were heat and freeze thawing.

Chisari *et al.*[4] reported of the rosette inhibitory factor which was thought to be a low density lipoprotein in the serum of HB-hepatitis. This rosette inhibitory factor had the same effect to normal lymphocytes. But the serum factors from patients with bladder cancer had no E-rosette suppressive effect to normal lymphocytes from healthy controls. It was said by Mortensen *et al.*[9] that purified human C-reactive protein (CRP) selectively connected with T lymphocytes and inhibited E-rosette

TABLE 4. Abrogation of Restorative Effect of Levamisole on E-Rosettes Forming Cells by Autochthonous Serum in Some Concentrations (Bladder Cancer Patients)

Lymphocytes donors Case	E-rosette forming cells (%)				
	Before levamisole treatment	After levamisole treatment	After treatment with autochthonous serum		
			50%	1%	0.5%
1	41	80	53	60	67
2	30	65	33	55	60
3	25	51	23	35	57
4	33	50	38	45	52
5	45	68	40	55	65

TABLE 5. Effect of Allogeneic Serum from Bladder Cancer Patients to the Restorated Lymphocytes by Levamisole

Lymphocytes donors Case	E-rosette forming cells (%)				
	Before levamisole treatment	After levamisole treatment	After treatment with allogeneic serum from bladder cancer patients		
			50%	1%	0.5%
1	41	80	64	71	75
2	30	65	60	66	60
3	25	51	51	49	48

TABLE 6. Effect of Allogeneic Serum from Bladder Cancer Patients to E-Rosette Formation of Lymphocytes from Normal Healthy Donors

Lymphocytes donors Case	E-rosette forming cells (%)	
	Before serum treatment	After treatment with 50% allogeneic serum from bladder cancer patients
1	57	50
2	59	65
3	63	60
4	70	64
5	67	65

TABLE 7. Effect of Levamisole Administration 150 mg/day for 3 Days of E and EAC-Rosette Forming Cells (Bladder Cancer Patients)

Lymphocytes donors Case	Before levamisole administration		After levamisole administration	
	E-rosette	EAC-rosette	E-rosette	EAC-rosette
	(%)		(%)	
1	41	27	78	14
2	51	18	69	13
3	41	20	68	18
4	30	23	65	20
5	45	25	70	22

formation. In our cases, there were no significant differences of E-rosette formation between CRP positive group and negative group. The mechanism responsible for suppression of E-rosette formation by the serum factors appears to be related to the impaired lymphocytes. Recently, the effects of levamisole *in vivo* have been shown to be beneficial in some cancer patients.[1,14] Levamisole may be useful in the treatment of various cancer patients as one of maneuvers of recovering the defective cellular immunity, whereas more investigations about such serum factors may be necessary.

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