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Effects of Cimetidine on Interleukin-2 Production by Peripheral Blood Lymphocytes in Advanced Ovarian Carcinoma*

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Abstract—*The present study was designed to elucidate the effect of cimetidine on CD4 and CD8 positive cells, interleukin-2 (IL-2) production and IL-2 receptor expression in peripheral blood lymphocytes (PBL) from patients with advanced ovarian carcinoma during the course of combination chemotherapy. The absolute number of CD8 (but not CD4) positive cells in PBL from patients with advanced ovarian carcinoma before surgery was significantly higher than that with benign ovarian tumor, while the IL-2 productivity was significantly lower. However, the IL-2 receptor expression was comparable to that with benign ovarian tumor. When a combination chemotherapy consisting of cisplatin, adriamycin and cyclophosphamide was given to these ovarian cancer patients, the IL-2 production was markedly depressed. If cimetidine was given with the combination chemotherapy, the inhibition of IL-2 production by chemotherapy was significantly diminished with a significant increase of CD4 positive cells. On the other hand, the IL-2 receptor expression was not affected by this treatment. When treatment with cimetidine was initiated after completion of chemotherapy, the depressed IL-2 production was restored to the level of control patients with benign ovarian tumor. The restoration seemed to result from an increase in proportion of CD4 positive cells. However, the expression of IL-2 receptor remained unchanged even if cimetidine was given.*

INTRODUCTION

IT HAS BEEN reported that immune dysfunction with increasing tumor burden are associated with a concomitant decline in the functional capacity of T lymphocytes [1, 2]. Previous investigations have demonstrated that clonal expansion of T lymphocytes after antigen or mitogen triggering requires the presence of a soluble mediator or interleukin [3–7]. In addition, such a factor also appears to be necessary for differentiation of precytotoxic T lymphocyte into cytotoxic T lymphocyte effector cells [8, 9]. Several lines of evidence support the notion that a single T cell-derived glycoprotein, termed interleukin-2 (IL-2), possesses both activities in man as well as experimental animals [10, 11]. It has also been reported that the slowing down or cessation of cytotoxic activity against tumor cells may be due to the absence or perturbation of IL-2 [12]. Recently, it has been shown that 50–60% of peripheral T lymphocytes are IL-2 producing cells and about 75% of $CD4^+$ cells have IL-2 producing capacity, whereas only about 15% of $CD8^+$ cells exhibited this potential [13]. In most patients with ovarian carcinoma, it is usual to give combination chemotherapy after surgery [14]. We have already reported that such combination chemotherapy suppressed the NK activity in all patients [15]. In addition, the suppressed NK activity was demonstrated to recover after treatment with cimetidine [16, 17]. It is well-known that IL-2, called T cell growth factor, is one of the most important immunoregulatory lymphokines elaborated by helper T-lymphocytes and NK cells. IL-2 is also a potent augmentor of NK cell tumoricidal activity [18, 19]. In a recent report, it has been shown that cimetidine enhanced, though moderately, the IL-2 production from peripheral blood lymphocytes (PBL) of the melanoma patients or normal PBL after phytohemagglutinin-P [PHA-P] stimulation [20]. Therefore, it seemed to be of practical importance to determine the effects of cimetidine on CD4 and CD8 positive cells, IL-2 production and receptor expression in PBL of ovarian cancer patients receiv-

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ing combination chemotherapy. In the present study, we now report inhibitory effects of combination chemotherapy on IL-2 production and its restoration by cimetidine.

MATERIALS AND METHODS

Patients

The study was made on 32 patients (median age, 48 years) with benign ovarian tumors and 38 patients (median age, 51 years) with advanced ovarian carcinoma including five stage IIc patients, 28 stage III patients and five stage IV patients. All the cancer patients received six courses of combination chemotherapy commencing about 2 weeks after surgery. Blood samples, before chemotherapy, were taken about 10 days after surgery. One course of combination chemotherapy consisted of cisplatin (30 mg/m^2/day i.v., on day 1), adriamycin (7 mg/m^2/day i.v., on days 1–5) and cyclophosphamide (140 mg/m^2/day i.v., on days 1–5). To determine the immediate effect of the combination chemotherapy on CD4 and CD8 positive cells, IL-2 production and receptor expression, the CD4 and CD8 positive cells, IL-2 production and receptor expression levels in PBL before chemotherapy were compared to those 3–5 days after the first course of combination chemotherapy. The effects on the CD4 and CD8 positive cells, IL-2 production and receptor expression when cimetidine was given with the combination chemotherapy was also compared to those when treated with chemotherapy alone. In addition, to determine effects of cimetidine on the CD4 and CD8 positive cells, IL-2 production and receptor expression after completion of chemotherapy, cimetidine was administered orally for more than 60 days after completion of chemotherapy and blood samples were collected before, 30 days and 60 days after treatment with cimetidine.

Immunotherapy

Cimetidine (Fijisawa Pharmaceutical Co. Ltd., Tokyo, Japan) was given orally 1.0 g/day during the course of chemotherapy or after completion of chemotherapy. Twenty stage III patients with less than 2 cm residual tumor after surgery were divided into cimetidine-treated and cimetidine-untreated groups. All these patients had good performance status after surgery.

Peripheral blood lymphocytes

Blood was collected by venipuncture into heparinized (10 U/ml of blood) tubes. Lymphocytes were separated by centifugation on a Ficoll–Hypaque gradient [21], washed three times with phosphate-buffered saline (0.15 M, pH 7.2), counted and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco Laboratories, New York, NY, USA) and 2 mM glutamine.

Determination of CD4 and CD8 positive cells

Immunofluorescence staining of the lymphoid cells with the monoclonal antibodies CD4 and CD8 (Ortho Pharmaceutical, Raritan, NJ) was performed with an indirect system using monoclonal antibody-containing supernatant in the first step and fluorescein-conjugated goat anti-mouse IgG_1 or fluorescein-conjugated goal anti-mouse IgG_2 in the second step. The reagents were first centrifuged at 100,000 ***g*** before use, and 10^6 target lymphocytes/well in microtiter plates were reacted with saturation levels of first and second reagents. Cells were stained on ice in the presence of 0.01% NaN_3. The stained cells were analyzed by flow cytometry (a Jasco Model FCS-1; Japan Spectroscopic Co. Ltd., Tokyo, Japan). Cells stained above the second-step background level (usually below 1%) were considered positive. Percentage positive cells were obtained by subtracting background values.

IL-2 production

PBL separated from heparinized blood by the Ficoll–Hypaque density gradient centrifugation method were suspended in RPMI 1640 medium supplemented with 5% FCS and 1% PHA in a concentration of 10^6 cells/ml, and cultured for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. The culture supernatant was collected and cryopreserved at −80°C. The IL-2 content of the supernatants was estimated as described by Gillis *et al.* [22], using the IL-2 dependent cell line CTLL-2 [23]. CTLL-2 cells are highly sensitive and specific to IL-2. IL-2 units were calculated using probit analysis [22] with reference to one batch of standard IL-2 (human recombinant IL-2; Biogen Co. Ltd., Geneva, Switzerland).

Measurement of IL-2 receptor expression

Radiolabeled IL-2 binding to whole cells was performed as described previously in detail [6]. All cells were prepared for the assay by centrifugation, followed by incubation at 37°C in IL-2 free RPMI 1640 medium (50 ml/l × 10^7 cells) for two 1-h intervals to promote dissociation and/or degradation of endogenously bound IL-2. These conditions were chosen based upon the dissociation rate constant previously determined for intact cells and isolated plasma membranes ($t_{1/2}$ for dissociation is 25 min) [6]. Serial dilutions of [^{3}H]leu,lys-IL-2 were incubated with cells (1 × 10^6 cells/0.2 ml) in RPMI 1640 medium, 1 mg/ml bovine serum albumin (BSA) at 37°C. After a 20 min incubation, cold (4°C) RPMI 1640-BSA (1 ml) was added and the cells centrifuged (9000 ***g***, 15 s). The supernatant containing the unbound fraction was removed and counted in a liquid scintillation counter. The cell pellet was resuspended in 100 μl cold RPMI 1640-BSA and centrifuged (9000 ***g***, 90 s)

through a 200 μl layer of a mixture of 84% silicone oil and 16% paraffin oil. The tips of the tubes containing the cell pellet were cut off and examined by liquid scintillation counting to determine the level of bound radioactivity. The calculated value of the number of binding sites per cell were obtained by Scatchard analysis of equilibrium binding data, after subtraction of the nonsaturable binding determined in the presence of a 150-fold molar excess of unlabeled IL-2. The lower limit of detection of receptor sites per cell was 200.

RESULTS

Absolute numbers of CD4 and CD8 positive cells, IL-2 production and receptor expression in PBL from patients with advanced ovarian carcinoma were evaluated before surgery and during the course of chemotherapy. The absolute number of the CD8 positive cells in PBL from patients with advanced ovarian carcinoma before surgery was significantly higher than in patients with benign ovarian tumors, while preoperative levels of the IL-2 production were significantly lower. However, no significant difference in the IL-2 receptor levels was observed (Table 1). Patients with advanced ovarian carcinoma were treated with combination chemotherapy after surgery. The combination chemotherapy has shown to depress profoundly the NK activity [17]. Thus, we attempted to determine the immediate effects of combination chemotherapy on these immune functions. Three to five days after the first course of combination chemotherapy, the absolute numbers of $CD4^+$ and $CD8^+$ cells in patients not treated with cimetidine remained unchanged. When cimetidine was administered with the combination chemotherapy, the absolute number of $CD4^+$ (but not $CD8^+$) cells was significantly increased (Table 2). Although the IL-2 production was markedly inhibited 3–5 days after the chemotherapy, the degree of the inhibition was significantly reduced by treatment with cimetidine (Fig. 1). However, the expression of IL-2 receptor was only slightly inhibited after the first course of combination chemotherapy, compared to the pretreatment level. Treatment with cimetidine tended to elevate (but not significantly) the expression of IL-2 receptor during the course of chemotherapy (Fig. 2). Following six courses of chemotherapy, the effects of giving cimetidine were examined. Although the absolute numbers of the CD4 and CD8 positive cells of patients not treated with cimetidine remained unchanged either 30 days or 60 days after completion of chemotherapy, the absolute number of $CD4^+$ (but not $CD8^+$) cells of patients treated with cimetidine was significantly increased at 60 days (but not 30 days) after completion of the chemo-

Table 1. Preoperative level of CD4 and CD8 positive cells, IL-2 production and receptor expression in PBL from patients with benign or malignant ovarian tumors

	Benign tumors	Malignant tumors
No. of cases	32	38
Median age (range)	48 (29–77)	51 (33–75)
$CD4^+$ cells*	1035 ± 152	1055 ± 243
$CD8^+$ cells	411 ± 139	564 ± 122‡
IL-2 production§	20.4 ± 8.1	11.9 ± 8.626‡
IL-receptor (%)	33.8 ± 12.0	26.2 ± 17.0

*Absolute number/μl.
†Mean ± S.D.
‡$P < 0.001$ (Student's *t*-test), compared to benign tumors.
§Units/ml.
Normal values (mean values) for $CD4^+$ cells, $CD8^+$ cells, IL-2 production and IL-2 receptor expression were 1030, 421, 19.7 and 42.1, respectively.

Table 2. Effects of combination chemotherapy and cimetidine on T cell subsets

Treatment group	*n*	Before chemotherapy*		After chemotherapy†	
		$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Chemotherapy alone	10	619 ± 356‡	510 ± 281	854 ± 450	512 ± 160
Chemotherapy plus cimetidine	10	702 ± 196	431 ± 198	1128 ± 371§	422 ± 162

*10 days after surgery.
†3–5 days after the first course of combination chemotherapy.
‡Absolute number of cells per μl. The mean ± S.D.
§$P < 0.01$ (Student's *t*-test), compared to before chemotherapy.

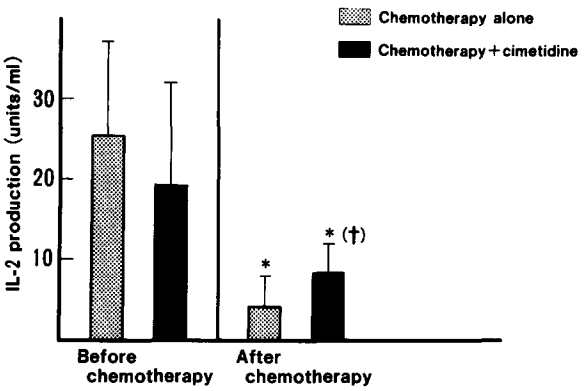

*Fig. 1. Effects of combination chemotherapy and cimetidine on IL-2 production. Each group consisted of seven patients. *P < 0.01, compared to before chemotherapy. †P < 0.05, compared to patients treated with chemotherapy alone. Bars show the mean ± S.D.*

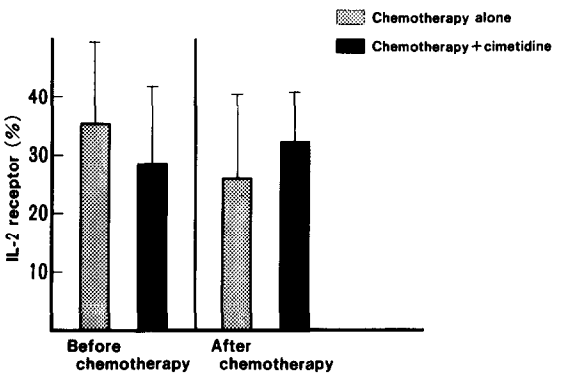

Fig. 2. Effects of combination chemotherapy and cimetidine on IL-2 receptor expression. Bars show the mean ± S.D.

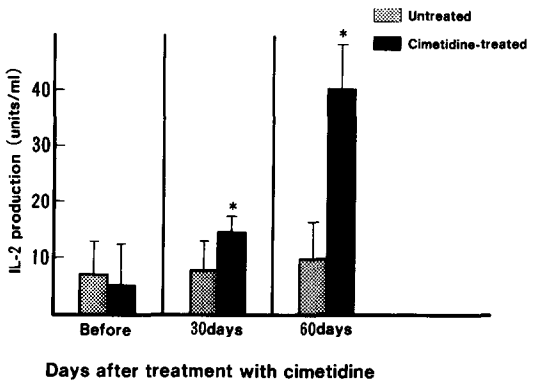

*Fig. 3. Changes of IL-2 production by PBL from patients treated with cimetidine after completion of combination chemotherapy. *P > 0.05, compared to before treatment with cimetidine and untreated group. Bars show the mean ± S.D.*

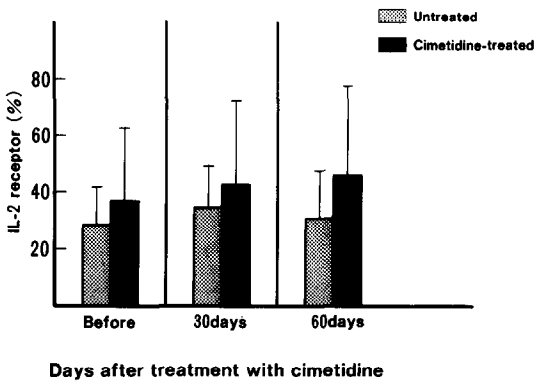

Fig. 4. Changes of IL-2 receptor expression in PBL from patients treated with cimetidine after completion of combination chemotherapy. Bars show the mean ± S.D.

therapy, compared to before treatment with cimetidine and untreated group (Table 3). Unless treatment with cimetidine was given after chemotherapy, the IL-2 production levels remained suppressed. By contrast, cimetidine induced about 3- and 8-fold increases in the pretreatment IL-2 production level at 30 days and 60 days, respectively (Fig. 3). On the other hand, even if cimetidine was administered after completion of the chemotherapy the expression of IL-2 receptor remained unchanged either 30 days or 60 days after treatment with cimetidine (Fig. 4).

DISCUSSION

Earlier reports indicated that IL-2 is involved in the development of cytotoxic T lymphocytes [24, 25] and that a host compromised by tumor has a defective cell-mediated immune response involving these cytotoxic T lymphocytes [23]. A significant decrease of the IL-2 production by PBL from patients with advanced ovarian carcinoma was accompanied with an increase of CD8 positive cells (suppressor/cytotoxic phenotype) (Table 1). Similar observations have also been made in patients with endometrial carcinoma [26] and in mouse with fibrosarcoma [5]. In previous studies, the existence of tumor-induced suppressor T cells that could inhibit cellular proliferation and IL-2 synthesis has been demonstrated [5, 27]. Thus, IL-2 production in patients with established cancer was considered

Table 3. Changes of T cell subsets in PBL from patients treated with cimetidine after completion of combination chemotherapy

Treatment	*n*		Before*	30 days	60 days
Untreated	10	$CD4^+$	782 ± 216†	831 ± 311	914 ± 275
		$CD8^+$	401 ± 127	384 ± 140	451 ± 219
Cimetidine-treated	10	$CD4^+$	837 ± 317	1037 ± 216	1386 ± 384‡
		$CD8^+$	481 ± 242	361 ± 195	410 ± 182

*3–5 days after completion of chemotherapy.
†Absolute number of cells per μl. The mean ± S.D.
‡$P < 0.01$ (Student's *t*-test), compared to before treatment with cimetidine and untreated group.

to be suppressed. Even though the IL-2 production was profoundly suppressed by the combination chemotherapy, the extent of inhibition was reduced by combination with cimetidine and proportion of CD4 positive cells (helper/inducer phenotype) was increased. Therefore, cimetidine seemed to have a significant protective effect to chemotherapy with respect to inhibition of IL-2 production. As described by Kikuchi *et al.* [17], cimetidine, a histamine type-2 antagonist, inhibits excessive suppressor T cell functions in patients with ovarian carcinoma.

Similarly, inhibition of suppressor cell function by cimetidine has been shown in a murine model [28]. Cimetidine appears to elicit the protective effect to chemotherapy on the IL-2 production through elimination of suppressor T cell functions. On the other hand, IL-2 receptor expression was not affected by chemotherapy, suggesting that cells capable of binding to IL-2 are relatively resistant to chemotherapy. In addition, we examined effects of cimetidine administered after completion of combination chemotherapy on the CD4 and CD8 positive cells, the IL-2 production and receptor expression. Unless cimetidine was given after completion of chemotherapy, these immunologic functions remained unchanged up to 60 days after completion of chemotherapy . However, when cimetidine was given after completion of chemotherapy, the IL-2 production was significantly enhanced 30 days and 60 days after completion of chemotherapy with an increase of CD4 positive cells. These results suggest that cimetidine may prove to be of clinical use as an immunostimulator for ovarian cancer patients with depressed IL-2 productivity receiving combination chemotherapy. However, in the present study we have demonstrated that the IL-2 receptor expression remained unchanged during tumor growth or the treatment course. This finding seems to result from a decrease of the IL-2 production that may be responsible for induction of IL-2 receptor expression and that cimetidine failed to prevent the decrease. The finding that IL-2 receptor expression remained unchanged is interesting in that receptor expression has been shown to be dependent either on the presence of soluble factors which are decreased during tumor growth or on the continued presence of a foreign antigen (tumor) [12]. Recently, Lotze *et al.* [29] revealed that high levels of IL-2 receptor positive lymphocytes were found in the peripheral blood of patients with malignant tumors receiving recombinant IL-2.

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