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Antitumor Effect of Interleukin 2 and Cyclophosphamide Administration

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Summary

EL4-bearing C57BL mice were treated cyclophosphamide and/or with IL2. Mice treated with cyclophosphamide alone survived significantly longer than nontreated mice, while IL2-treated mice did not. Mice treated with both cyclophosphamide and IL2 survived significantly longer than mice treated with cyclophosphamide alone. IL2 response of lymphocytes was markedly depressed in EL4-bearing mice. Lymphocytes from EL4-bearing mice treated with cyclophosphamide alone responded to IL2 significantly higher than those from mice treated with IL2 alone or those from nontreated mice. Lymphocytes from mice treated with both cyclophosphamide and IL2 responded to IL2 almost same as normal lymphocytes. Cyclophosphamide treatment protected the appearance of both suppressor factor that were found in EL4-bearing mice and inhibited IL2 response of lymphocytes.

These results suggested that combination therapy of cyclophosphamide and IL2 showed synergistic antitumor effect through the protection of IL2 responsiveness from suppression by suppressors.

‹Key words› Interleukin 2, Cyclophosphamide, Blastogenesis

Introduction

Immunodeficiencies in cancer patients are the results of depression of cytokine production such as interleukin 2 (IL2) which participates important roles in cellular interaction in the immune responses (1-3). This is the reason that administration of IL2 plays the leading part in the recent immunotherapy of cancer patients (4-6). However, suppressor cells or suppressor factors which suppress the immune responses are found in cancer patients (7-11). We are afraid whether such immunotherapy can actively play its immunopotentiating activity in the presence of these cells or factors. In this paper, we studied the effect of combination therapy with IL2 and cyclophosphamide which is effective to remove suppressor

hours and the uptake of ^{3}H-thymidine was determined as described above. The dpm of the normal lymphocytes in the co-culture was obtained from the following formula;

(dpm of normal lymphocytes)

= (dpm of the co-culture) - (dpm of added cell alone).

Determination of suppressor factor activity of serum. Normal lymphocytes were stimulated with ConA and blastogenic activity was determined in the culture medium contained EL4-bearing mouse serum. One tenth volume of serum collected from mice 14 days after EL4 graft was added to the normal lymphocyte suspension (1 x 10^6 cells/ml) and its suppressive effect on the blastogenic activities of normal lymphocytes to both ConA and IL2 were determined as described above.

Culture medium. Culture medium was RPMI 1640 medium supplemented with 10% fetal calf serum (Microbiological Associates, Maryland, Md.), 50 μg/ml of streptomycin and 50 units/ml of penicillin G.

Results

Effect of combination therapy of CY and IL2 on the survival days of EL4-bearing mice

Mice subcutaneously transplanted 1 x 10^5 of EL4 cells were treated with single injection of CY 3 days after EL4 graft and/or with intravenous injections of IL2 once a day for 7 days from 3 days after EL4 transplantation. The results are shown in Table 1. The mean survival days of mice treated with IL2 alone was 18.0 ± 0.9 days, while that of nontreated mice was 18.0 ± 0.9. No significant difference of survival days was observed between nontreated group and the group treated with IL2 alone. Mean survival days (23.3 ± 2.3) of mice treated with CY alone was significantly longer than that of nontreated mice ($P<0.01$ by student t-test). Mice treated with both CY and IL2 survived significantly longer (27.8 ± 3.4 days) than those of IL2-treated mice and of mice treated with CY alone ($P<0.01$ between the result of combination therapy and that of CY therapy). These results suggested the synergistic antitumor effect of combination therapy with CY and IL2.

Effect of CY and IL2 administration on the responsibility

of lymphocytes to IL2

CY was intraperitoneally injected into EL4-bearing mice 3 days after tumor graft. IL2 was intravenously injected into EL4-bearing mice 7 concecutive days from 3 days after tumor graft. Lymphocytes were prepared 14 days after EL4 graft and stimulated with IL2. As shown in Table 2, lymphocyte response to IL2 was markedly retarded in nontreated EL4-bearing mice. Treatment with IL2 alone showed no or a little effect on the responsiveness to IL2, whereas lymphocytes prepared from EL4-bearing mice treated with CY alone significantly recovered their IL2 responsiveness ($P<0.01$ by student t-test). Lymphocytes from CY- and IL2-treated mice showed almost same IL2 response as normal mouse lymphocytes. These results suggested that there are suppressor cells and suppressor factor in EL4-bearing mice which inhibit the IL2 response of lymphocytes and that the lymphocyte response to IL2 was recovered with CY treatment.

Suppression effect of EL4 ascites on normal lymphocyte response to IL2

Ascites was collected from mice 7 days after intraperitoneal transplantation of 1×10^6 EL4 cells. Normal lymphocytes were stimulated with IL2 and/or ConA with or without EL4 ascites. As shown in Table 3, EL4 ascites markedly suppressed the lymphocyte responses, suggesting that EL4 cells produced factor(s) suppressing lymphocyte response to IL2.

Effect of CY on the production of suppressor factor(s) in EL4-bearing mice

Sera were collected from mice 14 days after EL4 graft. Normal lymphocytes were stimulated with both IL2 and ConA in normal or EL4-bearing mouse seru. As shown in Table 4, serum collected from nontreated EL4-bearing mouse inhibited the normal lymphocyte response as compared with normal mouse serum ($P<0.05$ by student t-test) suggesting that the presence of suppressor factor(s) in EL4-bearing mouse serum. In contrast, lymphocyte response with serum collected from CY-treated tumor-bearing mice showed almost the same response as that with normal serum, while serum from IL2-treated EL4-bearing mouse showed suppression activity. These results suggested that the production of suppressor facto(s) by EL4 cells was inhibited by CY treatment.

cells.

Materials and Methods

Animals. Six to seven week old female C57BL/6 mice were used. Mice were purchased from Japan Clea Co. Ltd., Tokyo.

Tumor and tumor-bearing mice. Mouse leukemic EL4 cells were maintained as an ascites form in C57BL mice. EL4 cells were collected from the ascites 7 day after the inoculation of 1 x 10^6 EL4 cells, washed twice with RPMI 1640 medium (Nissui Kagaku Co. Ltd., Tokyo) and resuspended in the same medium at a concentration of 1 x 10^6 cells/ml. One tenth milliliter of the cell suspension was subcutaneously inoculated into C57BL mice and used as tumor-bearing mice.

Cyclophosphamide and interleukin 2 administration. EL4-bearing mice were intraperitoneally injected 100 mg/kg of cyclophosphamide (CY, Shionogi Co. Ltd., Tokyo) once 3 days after EL4 transplantation and were intravenously injected 10,000 units/mouse of human recombinant interleukin 2 (IL2, Shionogi Co. Ltd., Tokyo) once a day for 7 consecutive days from 3 days after EL4 graft.

Determination of in vivo antitumor effect. Antitumor effect was determined by the prolongation of survival of EL4-bearing mice.

Determination of responsiveness of lymphocytes to IL2. Spleen lymphocytes were collected as described previously (12) 14 days after the tumor graft. One tenth milliliter of lymphocyte suspension (1 x 10^6 cells/ml) was cultured with 500 units/ml of IL2 in the culture medium for 48 hours and, after incubation for another 24 hours with 0.5 μCi/ml of methyl-^{3}H-hymidine (2 Ci/mM, New England Nuclear Corp, Boston), the uptake of ^{3}H-thymidine into the lymphocytes was assayed with a liquid scintillation counter.

Determination of suppressor cell activity. Spleen lymphocytes collected from tumor-bearing mice were added to normal lymphocytes and their suppression of response of to concanavarin A (ConA, Shigma Chemical Co., St. Louis, Mo.) was determined as described previously (13). Briefly, 0.05 ml of normal lymphocyte suspension (2 x 10^6 cells/ml) and the same volume of EL4-bearing mouse lymphocytes (2 x 10^6 cells/ml) were mixed and stimulated with 5 ug/ml of ConA for 72

Effect of CY treatment on the suppressor cell activity in EL4-bearing mouse spleens

EL4-bearing mice were treated with CY 3 days after tumor graft. Lymphocytes were collected 14 days after EL4 graft and their suppressive effects on responses of normal lymphocytes to both ConA and IL2 were determined as described above. As shown in Table 5, normal lymphocytes co-cultured with CY-treated mouse lymphocytes responded to an almost normal level, while lymphocytes from nontreated EL4-bearing mice significantly suppressed the response of normal lymphocytes. These results suggested that CY treatment prevented the appearance of suppressor cells in EL4-bearing mice.

Discussion

Immunotherapies with interleukin 2 (IL2) and -interferon to cancer patients are being carried out because the immunodeficiencies in cancer patients are thought to results from the depression of those cytokines (1-3). However,there are many cases (5,14,15) of unsuccessful effect of the immunotherapy with IL2, although there are many successful reports (4-6). More recently, combination therapy with IL2 and lymphokine-activated killer (LAK) cells has been shown to have a therapeutic effect on cancer (16-23). However, in advanced stage of cancer, suppressor cells or suppressor factor(s) appear in the patients (7-11) and so that a useful effect of immunotherapy is decreased in the presence of these factors. In this study, we observed the appearance of suppressor cells and suppressor factor(s) in EL4-bearing mice and IL2 responsiveness was suppressed in these mice. Anti-tumor effect of IL2 was not found in these mice. We also found that synergistic antitumor effect by combination therapy of IL2 and cyclophosphamide (CY). The appearance of suppressor cells and suppressor factor(s) were inhibited and the responsiveness of lymphocytes to IL2 recovered in CY-treated EL4-bearing mice. Effect of CY in combination therapy with IL2 might have resulted from the recovery of IL2 responsiveness of LAK cells and cytotoxic cells through the inhibition of the appearance of suppressor cells and suppressor factor(s) although CY alone showed direct tumoricidal activity because EL4-bearing mice treated with CY alone survived significantly longer than nontreated tumor-bearing mice. In human cancer patients, sup-

pressor cells and suppressor factors are also found (7-11). In these patients, lymphocyte response to IL2 may be suppressed and immunotherapy with IL2 or with both IL2 and LAK cells should be carried out after the treatment for removal of such suppressors. One of the reasons of therapeutic failure of LAK cell transfusion into cancer patients (5,22) may be the loss of growth of transfused LAK cells, although the smaller number of transfused LAK cells than effective number may be another reason.

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Table I. Effect of CY and IL2 Administration on the Survival Days of EL4-bearing Mice

Treated with	Survial days	Mean ± SD
None	16, 17, 18, 18, 18	17.4 ± 0.9
IL2	17, 17, 17, 17, 18 18, 19, 19, 19, 19	18.0 ± 0.9
CY	21, 21, 22, 22, 22 23, 23, 25, 27, 27	23.3 ± 2.3*
CY + IL2	22, 24, 24, 26, 27, 28 28, 30, 30, 31, 31, 33	27.8 ± 3.4**

Mice subcutaneously received 1 x 10^5 of EL4 cells and were administered with single intraperitoneal injection of CY 3 days after EL4 graft and with intravenous injections of IL2 once a day for seven days from 3 days after EL4 graft.

*The survival days were significantly longer than those of nontreated mice ($P<0.01$ by student t-test).

**The survival days were significantly longer than those of nontreated mice and of CY-treated mice ($P<0.01$ by student t-test).

Table II. Effect of EL4-ascites on the Responsiveness of Normal Lymphocytes to IL2

Stimulation with		EL4-ascites	Uptake of ^{3}H-thymidine (dpm)
IL2	ConA		
+	-	-	11,725.5 ± 2,044.9
		+	3,583.0 ± 984.4*
-	+	-	12,893.8 ± 4,261.3
		+	2,701.2 ± 1,055.8*
+	+	-	23,302.7 ± 1,883.2
		+	4,604.8 ± 1,151.2*
-	-	-	358.9 ± 207.2
		+	409.4 ± 111.8

*$P<0.01$ by student t-test; the response was significantly less than each control without ascites.

Table III. Effect of CY and IL2 Administration on the Responsiveness of Lymphocytes to IL2

Lymphocytes from	Treatment of mice	^{3}H-thymidine uptake (dpm)
Normal mice	None	11,083.5 ± 1,313.7*
EL4-bearing mice at 2 weeks	None	2,394.0 ± 1,463.1
	CY	8,179.6 ± 1,773.4*
	IL2	3,701.9 ± 1,064.0
	CY + IL2	1,2457.9 ± 2,926.3*

Mice were subcutaneously transplanted with 1 x 10^5 EL4 cells. CY was intraperitoneally injected into mice 3 days after EL4 graft. IL2 was intravenously injected into mice 7 concecutive days from 3 days after tumor transplantation.
*$P<0.01$ by student t-test; the response was higher than that of nontreated EL4-bearing mouse lymphocytes.

Table V. Effect of CY on Suppressor Factor Activity in Serum of EL4-bearing Mice

Serum from	Blastogenic activity	
	dpm	% of control
Normal nontreated mice	25,531.3 ± 878.1	100.0 ± 3.4
EL4-bearing mice		
Nontreated	15,491.3 ± 443.1	60.7 ± 1.7*
CY-treated	23,401.0 ± 1,873.9	91.7 ± 7.3**
IL2-treated	16,656.5 ± 1,916.3	65.2 ± 7.5*

Normal mouse lymphocytes were mixed with serum of normal or EL4-bearing mice and stimulated with both ConA and IL2. Serum was collected 2 weeks after EL4 graft.

*The response was significantly less than that with normal mouse serum ($P<0.05$ by student t-test).

**The response was significantly higher than that with serum of nontreated EL4-bearing mice.

Table IV. Effect of CY Administration on the Suppressor Cell Activity

Added lymphocytes prepared from*	Treatment of mice	^{3}H-thymidine uptake (dpm)**
Normal mice	None	22,860.4 ± 2,159.9***
EL4-bearing mice at 2 weeks	None	6,822.7 ± 1,051.6
	CY	19,231.6 ± 1,407.3***

Mice were subcutaneously received 1 x 10^{5} EL4 cells. CY was intraperitoneally injected into mice 3 days after EL4 graft.

*Lymphocytes were added to normal mouse lymphocytes and the mixture was stimulated with both IL2 and ConA.

**dpm in this table = (dpm of the co-culture) - (dpm of the added cell culture alone)

***$P<0.01$ by student t-test; the response was significantly higher than that with nontreated EL4-bearing mouse lymphocytes.