Jpn. J. Cancer Res. 81, 1032–1038, October 1990

The *in vivo* Anti-tumor Effect of Human Recombinant Interleukin-6

Misa Kitahara,[1] Susumu Kishimoto,[1] Toshio Hirano,[2] Tadamitsu Kishimoto[3] and Masaji Okada[1]

[1]The Department of Medicine, Osaka University Hospital, 1-1-50, Fukushima, Fukushima-ku, Osaka 553, [2]Division of Molecular Oncology, Biomedical Research Center, Osaka University Medical School, 4-3-57, Nakanoshima, Kita-ku, Osaka 530 and [3]The Institute for Molecular and Cellular Biology, Osaka University, 1-3, Yamada-oka, Suita 565

Administration of recombinant interleukin-6 (IL-6) was found to induce *in vivo* generation of cytotoxic T lymphocytes (CTL) against syngeneic transplantable erythroleukemia (FBL-3) in lymph node cells and peritoneal exudate cells (PEC) in C57BL/6 mice. Furthermore, 15 out of 16 C57BL/6 mice injected with 5×10^6 viable FBL-3 cells survived on day 100 when they were treated with 5×10^4 U of recombinant IL-6 three times a day on days 1, 2, 3, 5, 7 and 9 after the inoculation of tumor cells (the cure rate was 94%). Cured mice could reject the tumor cells rapidly after the re-inoculation of a large number of live FBL-3 cells. In contrast, all normal mice died of tumor development by day 10. In these cured mice, FBL-3-specific $CD4^-8^+$ CTL cells were found to be generated in PEC, spleen and lymph node cells by either *in vivo* or *in vitro* re-stimulation with FBL-3 cells, but lymphokine-activated killer cells never developed. The results suggested that the anti-tumor effect of IL-6 was mediated by *in vivo* induction of tumor-specific CTL.

Key words: IL-6 — Anti-tumor effect — Cytotoxic T cells *in vivo* — Cure rate — FBL-3 tumor

The induction of CTL[4] *in vitro* requires the activation, proliferation and differentiation of CTL precursors and this process is regulated by several cytokines including IL-2.[1-6] *In vitro* studies utilizing recombinant cytokines have revealed that IL-4, IL-5 and IL-6 function as KHF(s).[7-11] However, little is known about the role of these cytokines in the *in vivo* induction of CTL and anti-tumor effect. Administration of rIL-2 together with LAK cells was shown to exert anti-tumor effect against pulmonary and hepatic metastases of a variety of murine tumors.[12,13] Recently, murine tumors with the IL-2 or IL-4 gene were shown to be rejected by the activation of either CTL or non-specific anti-tumor activity *in vivo*.[14,15] These results suggest the important roles of cytokines in the *in vivo* activation of anti-tumor effect.

IL-6 was originally identified as a B cell differentiation factor and its cDNA was cloned.[16] Subsequent studies have shown that IL-6 has a wide variety of biological functions as a myeloma growth factor, hepatocyte-stimulating factor, a multi-CSF and thrombopoietin.[17-21] IL-6 acts not only on B cells but also on T cells as a T cell growth and differentiation factor.[7-9,22-24] rIL-6 was demonstrated to function as a late-acting KHF in the differentiation of human and murine CTL *in vitro* in our previous study.[8] Therefore, in this study, we examined the effect of the *in vivo* administration of rIL-6 on the tumor rejection. The results demonstrate that IL-6 can augment the cure rate of mice bearing syngeneic FBL-3 tumor by the induction of the tumor-specific CTL *in vivo*.

MATERIALS AND METHODS

Reagents and antibodies Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co. Ltd. (Tokyo), concanavalin A (ConA) from Pharmacia Fine Chemicals (Uppsala), fetal calf serum (FCS, lot 508708) from Irvine Scientific (Santa Ana, CA). Anti-L3T4-, anti-Lyt 2.2 and anti-Thy 1.2 monoclonal antibodies were provided by Dr. K. Kuribayashi (Kyoto University, Kyoto), Dr. E. Nakayama (Nagasaki University, Nagasaki) and Dr. C. S. Henney (Immunex Co., Seattle, WA), respectively.[25-27] Human rIL-6 (5×10^6 U/mg protein) was produced in *Escherichia coli* and purified as described.[28] The rIL-6 contained less than 0.3 ng of endotoxin/mg.[29] Human rIL-2 (5×10^7 U/mg protein) was a gift from Dr. J. Hamuro (Ajinomoto Co., Kawasaki).[6]

Tumor cell lines and mice FBL-3, a Friend virus-induced erythroleukemia (C57BL/6 origin; $H\text{-}2^b$) was kindly provided by Dr. K. Kumagai (Tohoku University, Sendai). FBL-3 has been maintained by *in vivo* serial ip transplantation in syngeneic mice (for use as an *in vitro* and *in vivo* stimulator cell line). A mastocytoma cell line, P815-Y (DBA/2 origin; $H\text{-}2^d$), and EL-4 (C57BL/6

[3] To whom correspondence should be addressed.

[4] Abbreviations used in this paper: CTL, cytotoxic T lymphocytes; IL-6, interleukin-6; r, recombinant; MLTC, mixed lymphocyte tumor culture; KHF, killer helper factor; FBL-3, a Friend virus-induced erythroleukemia; MMC, mitomycin-C; PEC, peritoneal exudate cells; Spl., spleen; LAK, lymphokine-activated killer; NK, natural killer; C′, complement; mAb, monoclonal antibody.

origin; H-2^b) were provided by Dr. C. S. Henney and YAC-1, an NK-sensitive Moloney virus-induced lymphoma of A/Sn origin (H-2^a), provided by Dr. T. Ogura (Tokushima University, Tokushima). Tumor cell lines were maintained *in vitro* in RPMI 1640 medium (Flow Laboratories, Inc., McLean, VA) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5×10^{-5} *M* 2-mercaptoethanol.[8] Inbred and specific pathogen-free female C57BL/6 and Balb/c mice were obtained from Shizuoka Animal Center (Hamamatsu). All mice were maintained in isolator cages, manipulated in laminar flow hoods and used between the ages of 8 and 12 weeks.

Treatment of cells with antibodies and complement A total of 2×10^6/ml C57BL/6 spleen cells, mesenteric lymph node cells, and PEC were treated with an anti-L3T4 mAb (1/10 diluted), an anti-Lyt 2.2 mAb (1/40 diluted) or an anti-Thy1.2 mAb (1/20 diluted) for 15 min at 4°C, and then incubated with guinea pig C′ (1/10 diluted) for 45 min at 37°C as described.[8]

Treatment of syngeneic tumor-bearing mice with rIL-6 and rIL-2 rIL-6 and rIL-2 were stabilized in 2% filter-sterilized normal C57BL/6 mouse serum and diluted to 5×10^4 U/0.75 ml and 5×10^3 U/0.75 ml in Hanks' balanced salt solution (HBSS), respectively. HBSS containing an equivalent concentration of normal mouse sera was used as control injections. C57BL/6 host mice (sixteen mice per each group) were inoculated ip with 5×10^6 FBL-3 cells on day 0, and were injected ip with either 5×10^4 U rIL-6, 5×10^3 U rIL-2, 5×10^4 U rIL-6 and 5×10^3 U rIL-2, or HBSS on days 1, 2, 3, 5, 7 and 9 in three divided doses per day.

***In vitro* induction of cytotoxic T cells from cured mice** Mice cured of FBL-3 by administration of rIL-6 were killed on day 100. Then 3.5×10^6 spleen cells, mesenteric lymph node cells or PEC suspended in 2 ml of culture medium were cultured with 3.5×10^5 FBL-3_{MMC} in wells of a Linbro multi-well culture plate (No. 76-033-05).[8] After 5 days, cells were harvested and used as effector cells in a cytotoxicity assay. The percent specific cytolytic activity was measured by means of a microcytotoxicity assay, using ^{51}Cr-labeled FBL-3 as target cells.[30]

***In vivo* induction of cytotoxic T cells from cured mice** Mice cured of FBL-3 by administration of rIL-6 were re-inoculated ip with 3×10^7 viable FBL-3 cells. At various time intervals after FBL-3 immunization (4 to 21 days), animals were killed and the spleen, PEC, and mesenteric lymph node populations were obtained. Single cell suspensions from these populations were prepared as described.[2] These cells were tested for lytic activity.

***In vivo* induction of cytotoxic T cells against syngeneic tumor by administration of rIL-6** C57BL/6 mice were immunized ip with 1×10^8 MMC-treated syngeneic FBL-3 tumor cells, and were injected ip with 5×10^4 U of rIL-6, 5×10^3 U of rIL-2, or HBSS for 6 days. Ten days after immunization, mesenteric lymph nodes and PEC were harvested and tested for lytic activity against FBL-3.

Statistics Experimental data on cure rate in mice treated with lymphokines were statistically evaluated using the Kaplan-Meier test.[31, 32]

RESULTS

Systemic administration of rIL-6 induces *in vivo* CTL generation To study the role of IL-6 in the *in vivo* induction of CTL, rIL-6 was administered ip after immunization with mitomycin-treated FBL-3 cells. C57BL/6 mice were injected ip with 1×10^8 FBL-3_{MMC} followed by the administration of 5×10^4 U of rIL-6, 5×10^3 U of rIL-2 or both IL-6 and IL-2 for 6 days. Cytotoxic activities against FBL-3 cells in lymph node cells and PEC on day 10 were assessed. As shown in Fig. 1, *in vivo* generation of cytotoxic cells was observed in lymph node cells as well as in PEC and the effects of rIL-6 and rIL-2 were comparable. The cytotoxic activity was specific for FBL-3, and it was not observed against EL-4 tumor cells. The results demonstrate that rIL-6 can augment the *in vivo* induction of CTL against syngeneic tumor cells.

rIL-6 administered *in vivo* can cure FBL-3 leukemia To examine whether rIL-6 administered *in vivo* mediates an

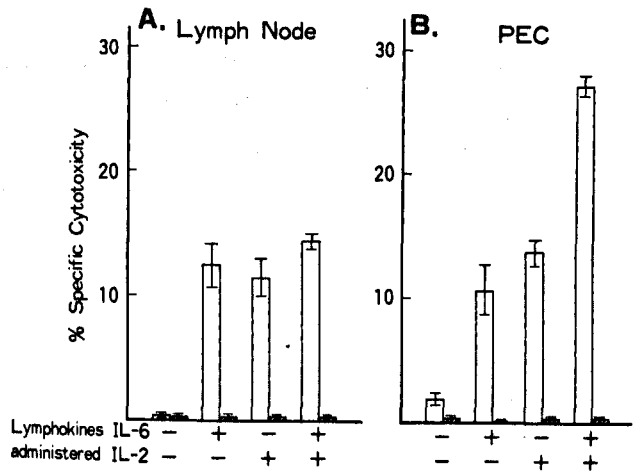

Fig. 1. Effect of rIL-6 on the *in vivo* generation of CTL against syngeneic FBL-3 tumor. Normal C57BL/6 mice were injected ip with 1×10^8 FBL-3_{MMC} cells on day 0, and then with 5×10^4 U of rIL-6, 5×10^3 U of rIL-2, both of 5×10^4 U of rIL-6 and 5×10^3 U of rIL-2, or HBSS on days 1, 2, 3, 5, 7 and 9 in three divided doses per day. Ten days after immunization, mesenteric lymph node cells (A) and PEC (B) were harvested and their cytotoxic activity was measured against FBL-3 cells (1×10^4; □) and EL-4 cells (1×10^4; ▨), at an E/T ratio of 130:1 and 20:1 in (A) and (B), respectively.

anti-tumor effect against FBL-3, C57BL/6 mice were injected ip with 5×10^6 viable FBL-3 cells on day 0 and were treated ip with 5×10^4 U of rIL-6 three times a day on days 1, 2, 3, 5, 7 and 9. As shown in Fig. 2, 94% of the mice (15 mice out of 16 mice) given rIL-6 were alive on day 100. The cure rate in mice injected with rIL-6 was significantly higher than that of 31% in mice injected with HBSS containing an equivalent concentration of normal mouse sera ($P<0.001$). The cure rate in mice treated with rIL-2 alone was 56% (9 mice out of 15 mice). The result indicates that IL-6 mediates an *in vivo* anti-tumor effect against FBL-3 tumor cells. In other repeated experiments, similar results were obtained (not shown).

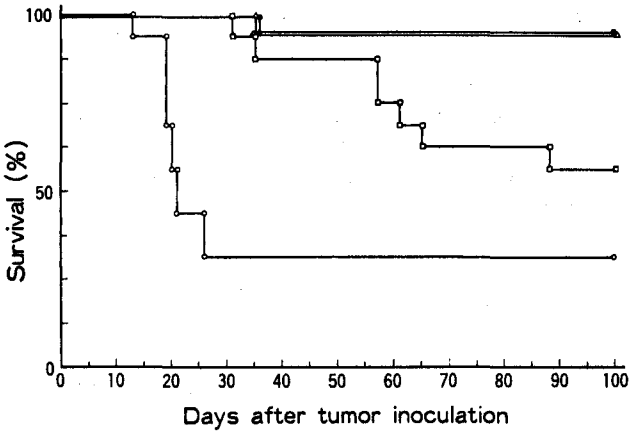

Fig. 2. An anti-tumor effect of rIL-6 by *in vivo* administration. Normal C57BL/6 mice (sixteen mice per group) were inoculated ip with 5×10^6 FBL-3 cells on day 0, and were injected ip with 5×10^4 U of rIL-6 (△), 5×10^3 U of rIL-2 (□), both of 5×10^4 U of rIL-6 and 5×10^3 U of rIL-2 (●), or HBSS (○) on days 1, 2, 4, 5, 7 and 9 in three divided doses per day. The cure rates in the groups were compared on day 100 after the tumor inoculation.

Table I. *In vitro* Induction of CTL against Syngeneic FBL-3 Tumor Cells from Spleen Cells in Cured Mice by the Administration of rIL-6

Cells		Treatment of effector cells	Specific cytotoxicity (%)	
Responder	Stimulator		E/T ratio	
Exp. I			30:1	6:1
Cured mice	$FBL\text{-}3_{MMC}$			
Spl.	20 ($\times10^5$)		1.1±0.3	1.5±0.2
	10		9.7±0.6	3.5±0.1
	3.5		45.6±1.8	13.2±0.8
	1		61.9±1.1	20.5±0.4
	0.35		29.1±1.1	9.0±1.2
	0		3.9±0.9	1.6±0.0
Normal mice	20		−0.7±0.0	1.1±1.1
Spl.	10		0.1±0.0	1.1±0.6
	3.5		0.2±0.1	0.7±0.1
	1		3.0±0.3	2.2±0.5
	0.35		5.3±0.1	2.6±0.9
	0		3.3±0.5	2.5±0.2
Exp. II				
Cured mice	$FBL\text{-}3_{MMC}$	—	24.2±2.2	
Spl.	3.5 ($\times10^5$)	C′	28.2±2.2	
		Anti-Thy 1.2+C′	0.6±0.2	
		Anti-L3T4 +C′	42.3±1.6	
		Anti-Lyt 2.2+C′	12.3±0.2	

(Exp. I) C57BL/6 mice cured of FBL-3 by administration of rIL-6 were killed on day 100. Spleen cells (3.5×10^6) from cured mice and normal C57BL/6 mice were cultured with various numbers of $FBL\text{-}3_{MMC}$ cells for 5 days. Cytotoxic activity against FBL-3 cells was assessed at effector-to-target ratios of 30:1 and 6:1. (Exp. II) 3.5×10^6 spleen cells from cured mice were cultured with 3.5×10^5 $FBL\text{-}3_{MMC}$ cells. After 5 days of culture, cells were harvested and these effector cells were treated with monoclonal anti-Thy1.2, anti-CD4 or anti-CD8 antibody together with complement as described in "Materials and Methods." After treatment, effector cells were assessed for cytotoxic activity at an effector-to-target ratio of 25:1.

T cells from mice cured by rIL-6 showed specific cytolytic activity against FBL-3 *in vitro* To determine whether specific immunity is operative in mice cured by the administration of IL-6, the spleen cells of these mice were cultured with $FBL\text{-}3_{MMC}$ under MLTC as described by Greenberg *et al.*[13] When 3.5×10^6 spleen cells from cured mice were cultured with $FBL\text{-}3_{MMC}$ ranging from 10×10^5 to 0.35×10^5 cells for 5 days, a significant cytotoxic activity against FBL-3 was generated (Table I). On the other hand, no cytotoxic cells were induced in normal spleen cells by the same stimulation. When effector cells generated from spleen cells in cured mice were treated with either anti-Thy1.2 or anti-CD8 mAb together with complement, cytolytic activity was significantly abrogated. However, the depletion of $CD4^+$ cells rather augmented the cytotoxic activity (Table I). The result suggests that $CD4^-8^+$ CTL played a role in the therapeutic efficacy of IL-6 against syngeneic FBL-3 tumor.

Cured mice rejected a large number of tumor cells To study in detail the role of IL-6 and CTL in cured mice, cured mice were re-inoculated with 3×10^7 viable FBL-3 cells. As shown in Fig. 3A, the maximal cytotoxic activity was observed in spleen cells, lymph node cells and PEC in cured mice on day 7 after re-inoculation of FBL-3 cells. All normal C57BL/6 mice died of FBL-3 tumors by day 10, and little cytotoxicity was generated in these mice (Fig. 3B). On the other hand, all the mice cured by the rIL-6 treatment rejected the same number of FBL-3 cells but not EL-4 tumor cells. Prolongation of survival time was not observed in these cured mice in comparison with normal C57BL/6 mice, when viable EL-4 tumor cells were inoculated (data not shown). These effector cells were found to be cytotoxic to FBL-3, but not YAC-1 (LAK-sensitive), EL-4, P815, and C57BL/6 spleen

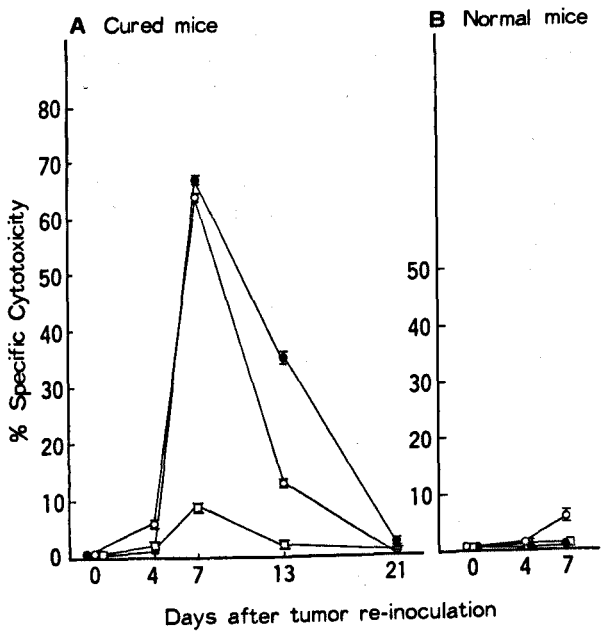

Fig. 3. Kinetics of *in vivo* CTL generation against FBL-3 cells by the re-inoculation of FBL-3 ip in mice cured by IL-6 treatment. (A) C57BL/6 mice cured by the administration of rIL-6 as shown in Fig. 2 were re-inoculated ip with 3×10^7 viable FBL-3 cells on day 100 after initial tumor injection. At intervals (on days 4, 7, 13, and 21) after FBL-3 re-immunization, animals were killed and spleen (□), PEC (●), and mesenteric lymph node (○) populations were harvested. (B) As a control, normal age-matched C57BL/6 mice were inoculated ip with 3×10^7 live FBL-3 cells, then killed on day 4 or 7 and the peripheral lymphoid populations were harvested. These populations were tested for lytic activity against ^{51}Cr-labeled FBL-3 cells at E/T ratios of 1000:1, 300:1, and 200:1, in spleens, lymph nodes, and PEC, respectively.

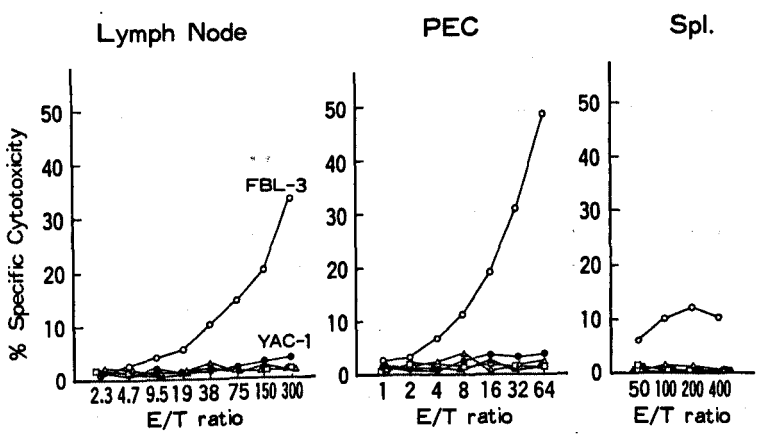

Fig. 4. Specificity of cytotoxic cells induced by *in vivo* re-stimulation with FBL-3 in mice cured by the administration of IL-6. C57BL/6 mice cured by the administration of rIL-6 as shown in Fig. 2 were re-inoculated ip with 3×10^7 live FBL-3 cells on day 100. On day 7 after FBL-3 re-immunization, animals were killed and the spleen, PEC, and mesenteric lymph node populations were harvested. These populations were tested for lytic activity against FBL-3 (○), YAC-1 (●), P815 (△), EL-4 (□) tumor cells and C57BL/6 spleen blast cells (▲) (stimulated with 2 μg/ml of ConA and 100 U/ml of rIL-2) at various E/T ratios.

Table II. Phenotype of Cytotoxic Effector Cells Induced by Re-stimulation of Cured Mice with Syngeneic FBL-3 Tumor Cells *in vivo*

Effector cells	Treatment of effector cells	Specific cytotoxicity (%)
Lymph node cells	—	20.5±0.2
	C′	19.6±0.2
	Anti-Thy 1.2+C′	−2.9±1.4
	Anti-Lyt 2.2+C′	4.2±1.4
	Anti-L3T4+C′	22.6±2.8
PEC	—	18.2±0.2
	C′	37.4±0.6
	Anti-Thy 1.2+C′	0.3±0.9
	Anti-Lyt 2.2+C′	2.5±0.1
	Anti-L3T4+C′	39.6±1.5

C57BL/6 mice cured by the administration of rIL-6 as shown in Fig. 2 were re-inoculated ip with 3×10^7 viable FBL-3 cells on day 100. On day 7 after FBL-3 re-inoculation, the animals were killed and mesenteric lymph nodes and PEC populations were harvested. These effector cells were treated with monoclonal anti-Thy1.2, anti-CD4 or anti-CD8 antibody together with complement. After treatment, effector cells were assessed for cytotoxic activity against FBL-3 cells at an effector-to-target ratio of 25:1.

blast cells even at high ratios of effector to target (Fig. 4). The results demonstrate that effector cells do not belong to the category of NK or LAK cells. Treatment of lymph node cells and PEC with anti-CD8 or anti-Thy1.2 mAb together with C′ almost completely abrogated the cytolytic activity. On the other hand, the depletion of $CD4^+$ cells had no effect on the cytotoxic activity (Table II). The results suggested that rIL-6 played a role in mediating an anti-tumor effect via the *in vivo* induction of $CD4^-8^+$ CTL.

DISCUSSION

The present study demonstrated that *in vivo* administration of rIL-6 augmented the cure rate of C57BL/6 mice inoculated with syngeneic FBL-3 tumor cells by the augmentation of the induction of tumor cell-specific CTL. Several lines of evidence supported this conclusion. (i) When spleen and lymph node cells from mice cured by rIL-6 were cultured with $FBL\text{-}3_{MMC}$ *in vitro*, $CD4^-8^+$ CTL against FBL-3 were generated. No CTL were generated from spleen and lymph node cells in normal mice. (ii) Effector CTL ($CD4^-8^+$) but not LAK cells were generated *in vivo* from PEC, spleen and lymph node cells in cured mice by the re-inoculation of FBL-3 cells. On the other hand, the inoculation of the same number of FBL-3 cells failed to induce CTL in normal C57BL/6 mice. (iii) rIL-6 was found to inhibit *in vitro* proliferation of some tumors.[33] The proliferation of FBL-3 cells *in vitro*, however, was not inhibited by the addition of rIL-6 ranging from 0.1 U/ml to 10^5 U/ml, even in the presence of rIL-2 (data not shown). Moreover, no direct cytotoxic effect of rIL-6 could be demonstrated on FBL-3 cells in a ^{51}Cr 4-h release assay. Interferon γ and tumor necrosis factor were shown to increase the sensitivity of target tumor cells to cytotoxic cells.[34–36] In contrast, the sensitivity of FBL-3 target cells to cytotoxic cells was not increased by rIL-6 at concentrations ranging from 0.1 U/ml to 5×10^4 U/ml for 48 h (data not shown). These results indicated that the anti-tumor effect of rIL-6 was not mediated by a direct cytotoxic and/or cytostatic effect on FBL-3 tumor cells.

Murine plasmacytomas and mammary adenocarcinomas transfected with murine IL-4 cDNA were found to generate inhibitory activity for *in vivo* tumor growth.[15] The anti-tumor effect of IL-4, however, was suggested to be mediated by an inflammatory infiltrate composed of macrophages and eosinophils, in contrast with the anti-tumor effect of IL-6 via CTL induction in the present study. It has not been elucidated whether systemic administration of IL-4 could be an efficient means for the eradication of tumors or not. Transfection of poorly immunogenic colon tumors with the gene for murine IL-2 was also found to elicit an anti-tumor effect by the induction of tumor-specific CTL.[14] IL-2 provided effectively by the transfected tumor cells was suggested to generate the CTL against poorly immunogenic tumors. In contrast, tumor-associated antigens encoded by the viral gene in immunogenic FBL-3 tumor cells could be recognized by helper T cells and CTL were elicited.[37] Actually, the anti-leukemia effect of high-dose IL-2 (5×10^4 U/day) even without the administration of immunocompetent cells was shown in mice bearing FBL-3 tumor.[13] Depletion of host T cells, either $CD4^+$ or $CD8^+$, but not $NK\text{-}1.1^+$ cells, reduced the therapeutic efficacy of IL-2.[38] In this study, it was not examined whether $CD4^+$ T cells were required for an anti-tumor effect of IL-6.

Maximal *in vitro* generation of FBL-reactive $CD8^+$ CTL and the *in vivo* therapeutic activity of this subset were shown to be dependent on $CD4^+$ helper T cells.[38, 39] The addition of rIL-6 to MLTC including unprimed C57BL/6 splenic $CD4^-8^+$ T cells and $FBL\text{-}3_{MMC}$ induced the generation of CTL *in vitro* against FBL-3 cells (data not shown). Furthermore, rIL-6 even when administered one day after the inoculation of $FBL\text{-}3_{MMC}$ cells induced the generation of tumor-specific CTL *in vivo* in the present study. These results suggest that IL-6 functions as a KHF in the differentiation of CTL *in vivo* as well as *in vitro*.[8] However, other possibilities might be considered: (i) IL-6 may have the activity to augment the expression of MHC class I antigens and tumor-associated antigens

required for the recognition of tumor-specific CTL, as demonstrated by Lurquin *et al.*[40] This possibility, however, is unlikely, since cytotoxic activity of FBL-3-reactive CTL against FBL-3 cells stimulated with rIL-6 *in vitro* was not augmented. (ii) IL-6 may induce CTL generation via the activation of helper T cells *in vivo*.

Three distinct factors (IL-4, IL-5 and IL-6) were shown to be involved in the generation of the *in vitro* CTL.[7-11] Therefore, it will be of interest to investigate whether IL-4 or IL-5 can augment the *in vivo* CTL generation and the anti-tumor effect induced by rIL-6. An anti-tumor activity of IL-6 mediating reduction in the number of micrometastases from sarcomas and adenocarcinoma has been reported by Mule *et al.*[41] It will be of interest to examine whether the administration of rIL-6 can be efficient for the eradication of poorly immunogenic tumors by the induction of CTL as well as for the eradication of immunogenic tumors. An approach utilizing poorly immunogenic tumors transfected with the IL-6 gene may be useful for elucidating this problem.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture. The authors wish to thank Drs. Y. Akiyama and J. Hamuro (Ajinomoto Co. Inc., Kawasaki), for providing rIL-6. We also thank Miss T. Imamura, Y. Fujii and K. Kubota for their secretarial assistance.

(Received May 28, 1990/Accepted August 3, 1990)

REFERENCES

1) Plate, J. M. D. Soluble factors substitute for T-T cell collaboration in generation of T killer lymphocytes. *Nature*, **260**, 329–331 (1976).
2) Okada, M., Klimpel, G. K., Kuppers, R. C. and Henney, C. S. The differentiation of cytotoxic T cells *in vitro*. I. Amplifying factor(s) in the primary response is Lyt1 cell-dependent. *J. Immunol.*, **122**, 2527–2533 (1979).
3) Kern, D. E., Gillis, S., Okada, M. and Henney, C. S. The role of interleukin-2 (IL-2) in the differentiation of cytotoxic T cells; the effect of monoclonal anti-IL-2 antibody and absorption with IL-2 dependent T cell lines. *J. Immunol.*, **127**, 1323–1328 (1981).
4) Raulet, D. H. and Bevan, M. J. A differentiation factor required for the expression of cytotoxic T-cell function. *Nature*, **296**, 754–757 (1982).
5) Kaieda, T., Okada, M., Yoshimura, N., Kishimoto, S., Yamamura, Y. and Kishimoto, T. A human helper T cell clone secreting both killer helper factor(s) and T cell-replacing factor(s). *J. Immunol.*, **129**, 46–51 (1982).
6) Okada, M., Yoshimura, N., Ichimori, Y., Kishimoto, S., Nakai, S., Nishino, N. and Kishimoto, T. Immunologic and molecular characterization of T cell derived T cell activating factor. *J. Immunol.*, **136**, 1288–1294 (1986).
7) Takai, Y., Wong, G. G., Clark, S. C., Burakoff, S. J. and Herrmann, S. H. B cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes. *J. Immunol.*, **140**, 508–512 (1988).
8) Okada, M., Kitahara, M., Kishimoto, S., Matsuda, T., Hirano, T. and Kishimoto, T. IL-6/BSF-2 function as a killer helper factor in the *in vitro* induction of cytotoxic T cells. *J. Immunol.*, **141**, 1543–1549 (1988).
9) Uyttenhove, C., Coulie, P. G. and Van Snick, J. T cell growth and differentiation induced by interleukin-HP1/IL-6, the murine hybridoma/plasmacytoma growth factor. *J. Exp. Med.*, **167**, 1417–1427 (1988).
10) Widmer, M. B. and Grabstein, K. H. Regulation of cytolytic T-lymphocyte generation by B-cell stimulatory factor. *Nature*, **326**, 795–798 (1987).
11) Takatsu, K., Kikuchi, Y., Takahashi, T., Honjo, T., Matsumoto, M., Harada, N., Yamaguchi, N. and Tominaga, A. Interleukin 5, a T cell-derived B-cell differentiation factor also induced cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA*, **84**, 4234–4238 (1987).
12) Lafreniere, R. and Rosenberg, S. A. Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. *Cancer Res.*, **45**, 3735–3741 (1985).
13) Thompson, J. A., Peace, D. J., Klarnet, J. P., Kern, D. E., Greenberg, P. D. and Cheever, M. A. Eradication of disseminated murine leukemia by treatment with high-dose interleukin 2. *J. Immunol.*, **137**, 3675–3680 (1986).
14) Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Volgelstein, B. and Frost, P. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell*, **60**, 397–403 (1990).
15) Tepper R. I., Pattengale, P. K. and Leder, P. Murine interleukin-4 displays potent anti-tumor activity *in vivo*. *Cell*, **57**, 503–512 (1989).
16) Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Matsui, H., Takahara, Y., Taniguchi, T. and Kishimoto, T., Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature*, **324**, 73–76 (1986).
17) Kishimoto, T. and Hirano, T. Molecular regulation of B lymphocyte response. *Ann. Rev. Immunol.*, **6**, 485–512 (1988).
18) Hirano, T. and Kishimoto, T. Interleukin-6. *Hand. Exp. Pharmacol.*, **95**, 633–665 (1990).
19) Van Damme, J. H., Opdenakker, G., Simpson, R. J., Rubira, M. R., Cayphas, S., Vink, A., Pilliau, A. and

Snick, J. V. Identification of the human 26-kd protein, interferon β_2 (IFN β_2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. *J. Exp. Med.*, **165**, 914–919 (1987).
20) Poupart, P., Vandenebeele, P., Cayphas, S., Snick, J. V., Haegeman, G., Kruys, V., Fiers, W. and Content, J. B cell growth modulating and differentiating activity of recombinant human 26-kd protein (BSF-2, HuIFN-β_2, HPGF). *EMBO J.*, **6**, 1219–1224 (1987).
21) Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoka, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. and Kishimoto, T. Autocrine generation and essential requirement of BSF-2/IL-6 for human multiple myelomas. *Nature*, **332**, 83–85 (1988).
22) Garman, R. D., Jacobs, K. A., Clark, S. C. and Raulet, D. H. B-cell-stimulatory factor 2 (β_2 interferon) functions as a second signal for interleukin 2 production by mature murine T cells. *Proc. Natl. Acad. Sci. USA*, **84**, 7629–7633 (1987).
23) Lotz, M., Jirik, F., Kabouridis, P., Tsoukas, C., Hirano, T., Kishimoto, T. and Carson, D. A. BSF-2/IL-6 is a costimulant for human thymocytes and T lymphocytes. *J. Exp. Med.*, **167**, 1253–1258 (1988).
24) Le, J., Fredrickson, G., Reis, L. F. L., Diamantstein, T., Hirano, T., Kishimoto, T. and Vilcek, J. Interleukin 2-dependent and interleukin 2-independent pathways of regulation of thymocyte function by interleukin 6. *Proc. Natl. Acad. Sci. USA*, **85**, 8643–8647 (1988).
25) Dialynas, D. P., Wilde, D. B., Marrack, P., Pierres, A., Wall, K. A., Harran, W., Otten, G., Loken, M. K., Pierres, M., Kappler, J. and Fitch, F. W. Characterization of the murine antigenic determinant, designated L3T4, recognized by monoclonal antibody GK 1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.*, **74**, 29–56 (1983).
26) Nakayama, E. and Uenaka, A. Effect of *in vivo* administration of Lyt antibodies; Lyt phenotype of T cells in lymphoid tissues and blocking of tumor rejection. *J. Exp. Med.*, **161**, 345–355 (1985).
27) Ledbetter, J. A. and Herzenberg, L. A. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.*, **47**, 63–90 (1979).
28) Tonouchi, N., Oouchi, N., Kashima, N., Kawai, M., Nagase, K., Okano, A., Matsui, H., Yamada, K., Hirano, T. and Kishimoto, T. High-level expression of human BSF-2/IL-6 cDNA in *Escherichia coli* using a new type of expression-preparation system. *J. Biochem.*, **104**, 30–34 (1988).
29) Takatsuki, F., Okano, A., Suzuki, C., Chieda, R., Takahara, Y., Hirano, T., Kishimoto, T., Hamuro, J. and Akiyama, Y. Human recombinant interleukin 6/B cell stimulatory factor 2 (IL-6/BSF-2) augments murine antigen-specific antibody responses *in vitro* and *in vivo*. *J. Immunol.*, **141**, 3072–3077 (1989).
30) Okada, M., Yoshimura, N., Kaidea, T., Yamamura, Y. and Kishimoto, T. Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. *Proc. Natl. Acad. Sci. USA*, **78**, 7717–7721 (1981).
31) Peto, R., Pike, M., Armitage, P., Breslow, N., Cox, D., Howard, S., Mantel, N., McPherson, K., Peto, J. and Smith, P. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. *Br. J. Cancer*, **35**, 1–39 (1977).
32) Eberlein, T. J., Rosenstein, M., Spiess, P., Wesley, R. and Rosenberg, S. A. Adoptive chemoimmunotherapy of a syngeneic murine lymphoma with long-term lymphoid cell lines expanded in T cell growth factor. *Cancer Immunol. Immunother.*, **13**, 5–13 (1982).
33) Chen, L., Mory, Y., Zilberstein, A. and Revel, M. Growth inhibition of human breast carcinoma and leukemia/lymphoma cell lines by recombinant interfereron-β_2. *Proc. Natl. Acad. Sci. USA*, **85**, 8037–8041 (1988).
34) Bukowski, J. F. and Welsh, R. M. Interferon enhances the susceptibility of virus infected fibroblasts to cytotoxic T cells. *J. Exp. Med.*, **161**, 257–262 (1985).
35) Stotter, H., Wiebke, E. A., Tomita, S., Belldegrun, A., Topalian, S., Rosenberg, S. A. and Lotze, M. T. Cytokines alter target cell susceptibility to lysis II. Evaluation of tumor infiltrating lymphocytes. *J. Immunol.*, **142**, 1767–1773 (1989).
36) Bialas, T., Kolitz, J., Levi, E., Polivka, A., Oez, S., Miller G. and Welte, K. Distinction of purified human natural killer cytotoxic factor from recombinant tumor necrosis factor and recombinant human lymphotoxin. *Cancer Res.*, **48**, 891–898 (1988).
37) Klarnet, J. P., Kern, D. E., Okuno, K., Holt, C., Lilly, F. and Greenberg, P. D. FBL-reactive $CD8^+$ cytotoxic and $CD4^+$ helper T lymphocytes recognize distinct Friend murine leukemia virus-encoded antigens. *J. Exp. Med.*, **169**, 457–467 (1989).
38) Peace, D. J. and Cheever, M. A. Toxicity and therapeutic efficacy of high-dose interleukin 2. *In vivo* infusion of antibody to NK-1.1 attenuates toxicity without compromising efficacy against murine leukemia. *J. Exp. Med.*, **169**, 161–173 (1989).
39) Kern, D. E., Klarnet, J. P., Jensen, M. C. V. and Greenberg, P. D. Requirement for recognition of class II molecules and processed tumor antigen for optimal generation of syngeneic tumor-specific class I-restricted CTL. *J. Immunol.*, **136**, 4303–4310 (1986).
40) Lurquin, C., Van Pel, A., Maiame, B., De Plaen, E., Szikora, J-P., Janssens, C., Reddehase, M. J., Lejeune, J. and Boon, T. Structure for the gene coding for tum^- antigen P91A; a peptide encoded by the mutated exon is recognized with L^d by cytolytic T cells. *Cell*, **58**, 293–303 (1989).
41) Mule, J. J., McIntosh, J. K., Jablons, D. M. and Rosenberg, S. A. Antitumor activity of recombinant interleukin 6 in mice. *J. Exp. Med.*, **171**, 629–636 (1990).