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

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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 x 10^4 viable FBL-3 cells survived on day 100 when they were treated with 5 x 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 viable 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 in vitro requires the activation, proliferation and differentiation of CTL precursors and this process is regulated by several cytokines including IL-2. In vitro studies utilizing recombinant cytokines have revealed that IL-4, IL-5 and IL-6 function as KHF(s). However, little is known about the role of these cytokines in the in vivo induction of CTL and anti-tumor effect. Administration of IL-2 together with LAK cells was shown to exert anti-tumor effect against pulmonary and hepatic metastases of a variety of murine tumors. 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. 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. 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. IL-6 acts not only on B cells but also on T cells as a T cell growth and differentiation factor. IL-6 was demonstrated to function as a late-acting KHF in the differentiation of human and murine CTL in vitro in our previous study. 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. Human rIL-6 (5 x 10^6 U/mg protein) was produced in Escherichia coli and purified as described. The rIL-6 contained less than 0.3 ng of endotoxin/mg. Human rIL-2 (5 x 10^7 U/mg protein) was a gift from Dr. J. Hamuro (Ajinomoto Co., Kawasaki).

Tumor cell lines and mice FBL-3, a Friend virus-induced erythroleukemia (C57BL/6 origin; H-2b) 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-2b), and EL-4 (C57BL/6 origin; H-2b) were obtained from Dr. K. Kumagai (Tohoku University, Sendai).
Anti-tumor Effect of IL-6 by CTL Induction in vivo

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 $1 \times 10^8$ FBL-3MMC followed by the administration of $5 \times 10^4$ U of rIL-6, $5 \times 10^3$ 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
anti-tumor effect against FBL-3, C57BL/6 mice were injected ip with $5 \times 10^6$ viable FBL-3 cells on day 0 and were treated ip with $5 \times 10^4$ 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 $5 \times 10^6$ FBL-3 cells on day 0, and were injected ip with $5 \times 10^4$ U of rIL-6 (△), $5 \times 10^3$ U of rIL-2 (□), both of $5 \times 10^4$ U of rIL-6 and $5 \times 10^3$ 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

| Exp. | Cells         | Treatment of effector cells | Specific cytotoxicity (%) | E/T ratio |
|------|---------------|-----------------------------|---------------------------|-----------|
|      | Responder     | Stimulator                  |                           | 30:1      | 6:1       |
|      | Cured mice    | FBL-3MMC                    |                           |           |           |
|      | Spl.          | 20 ($\times 10^5$)          |                           | 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       | FBL-3MMC                    |                           |           |           |
|      | Cured mice    |                             |                           |           |           |
|      | Spl.          | 3.5 ($\times 10^5$)         |                           |           |           |
|      |               | Anti-Thy 1.2+C'             |                           | 24.2±2.2  | 28.2±2.2  |
|      |               | Anti-L3T4+C'                |                           | 0.6±0.2   |           |
|      |               | Anti-Lyt 2.2+C'             |                           | 42.3±1.6  |           |
|      |               |                             |                           | 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 ($3.5 \times 10^6$) from cured mice and normal C57BL/6 mice were cultured with various numbers of FBL-3MMC 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) $3.5 \times 10^6$ spleen cells from cured mice were cultured with $3.5 \times 10^5$ FBL-3MMC 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-3MMC under MLTC as described by Greenberg et al. When 3.5 \times 10^6 spleen cells from cured mice were cultured with FBL-3MMC ranging from 10 \times 10^3 to 0.35 \times 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^+ 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 \times 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 cells.
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 i.p. with 5 × 10³ 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 mAb 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.

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-3MC 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. 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 10⁵ 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 ⁵¹Cr 4-h release assay. Interferon γ and tumor necrosis factor were shown to increase the sensitivity of target tumor cells to cytotoxic cells. 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 × 10⁴ 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.

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|                | Anti-Lyt 2.2 + C'           | 4.2 ± 1.4                |
|                | Anti-L3T4 + C'              | 22.6 ± 2.8               |
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|                | Anti-L3T4 + C'              | 39.6 ± 1.5               |

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required for the recognition of tumor-specific CTL, as demonstrated by Lurquin et al.10 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.

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