Study of the Effect of Lithium on Lymphokine-Activated Killer Cell Activity and Its Antitumor Growth (43509)

YIYUAN WU[1] AND DONGPO CAI

Department of Immunology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing 100021, People's Republic of China

Abstract. **The *in vitro* effect of lithium on lymphokine-activated killer cell (LAK) activity and its *in vivo* antitumor growth were observed. LAK activity was enhanced when LiCl was added during LAK cell induction, and this enhancement was observed both in human peripheral blood mononuclear cell and in mouse splenocytes used as LAK precursors. Cholera toxin, which can increase intracellular levels of cAMP, decreased LAK cell activity. However, lithium partially reversed this inhibitory effect, indicating that lithium increased LAK cell activity by decreasing cAMP levels. D-Sphingosine, an inhibitor of protein kinase C, and EGTA, a calcium chelator, both inhibited the LAK cell activity. However, their inhibitory effects could not be reversed by lithium because lithium was added in the culture in combination with one of these inhibitors during LAK cell induction. By using slot blot analysis, the effect of lithium on the expression of tumor necrosis factor-α mRNA of LAK cells was analyzed. Lithium increased the level of tumor necrosis factor-α mRNA when both lithium and interleukin 2 were added to induce LAK cells. The *in vivo* antitumor effect of lithium has also been studied. Using a mouse melanoma experimental model, the effect of lithium on tumor growth was also observed. Both lithium alone and interleukin 2/LAK had an antitumor effect, whereas the treatment of interleukin 2/LAK in combination with lithium had the strongest inhibitory effect on tumor growth, since this treatment resulted in reduction of tumor size and prolongation of survival in tumor-bearing mice. Therefore, it is hopeful that lithium can be used as a new immunomodulator for cancer immunotherapy and immune diseases.**

[P.S.E.B.M. 1992, Vol 201]

Our previous studies indicated that lithium enhanced interleukin (IL) 2 production by peripheral blood mononuclear cells (PBMC) and its mechanism involved a decrease in intracellular cAMP levels (1). However, little is known about an enhancing effect of lithium on lymphokine-activated killer (LAK) cell activity. Furthermore, it also would be interesting to know whether lithium has any antitumor action. The aim of this study was to explore lithium as a promising immunomodulator to augment LAK cell activity *in vitro*. The antitumor growth effect by lithium was also studied *in vivo* using a murine experimental model.

Materials and Methods

Animals. C57BL/6 mice, 8–12 weeks-old and weighing 18–22 g, were obtained from the Animal Center of the Chinese Academy of Medical Sciences.

Reagents. Lithium chloride (LiCl) was purchased from the Beijing Chemical Factory. Recombinant IL-2 was obtained from the Cetus Co. Cholera toxic (CT), EGTA, and D-sphingosine were purchased commercially from Sigma Chemical Co. [α-^{32}P]dCTP was purchased from Dupont Co. [^{125}I]iododeoxyuridine was obtained from the Institute of Nuclear Power, Chinese Academy of Sciences. The tumor necrosis factor (TNF)-α cDNA fragment, a 1.38-kb *Hin*dIII/*Bam*HI digested fragment of pAW 711 (a plasmid), was obtained from American Type Culture Collection.

Preparation of PBMC and Induction of LAK Cells. PBMC were separated from the heparinized venous blood of healthy adult donors by centrifugation on

[1] To whom requests for reprints should be addressed at Department of Immunology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing 100021, People's Republic of China.

Received January 14, 1992. [P.S.E.B.M. 1992, Vol 201]
Accepted June 23, 1992.

0037-9727/92/2013-0284$3.00/0

Ficoll-Hapaque. After three washes, the cells were adjusted to the optimal concentration with RPMI 1640 medium containing 10% new born calf serum (NCS) and added to 24-well plates. The PBMC were cultured with or without stimulants, such as IL-2, lithium, CT, EGTA, or D-sphingosine, under various conditions at 37°C in 5% CO_2 for 4 days. Cells were than collected and resuspended at a density of 8×10^6/ml in 15% NCS RPMI 1640 as effector cells.

LAK Cell Cytotoxicity Assay. LAK cell activity was assayed using [^{125}I]iododeoxyuridine release according to the method described by Cohen *et al.* (2), with some modification. The Eca 109 target cells (human esophageal carcinoma cell line) were harvested at log phase and resuspended at a density of $1–2 \times 10^6$/0.5 ml with 15% NCS RPMI 1640. After adding 3 μCi of [^{125}I]iododeoxyuridine, cells were cultured at 37°C for 2 hr and then washed, and the cell concentration was adjusted to 2×10^5/ml. Effector cells and target cells (effector cell to target cell ratio, 80:1) were added to round flatbottom 96-well tissue culture plates (in a total volume of 150 μl) and cultured at 37°C in 5% CO_2 for 20 hr. 1.7% Trypsin (35 μl) and 0.1% DNAase I (15 μl) were added to each well with gentle shaking. The cells were kept at 37°C for 30 min, and then centrifuged at 1000 rpm for 5 min; supernatant (100 μl) from each well was taken for counting the cpm. The percentage of lysis was calculated for LAK cell activity.

$$\% \text{ Cytolysis} = \frac{\text{cpm of experimental group} - \text{cpm of } SR}{\text{cpm of } MR - \text{cpm of } SR} \times 100 \quad [1]$$

with *SR* as spontaneous release and *MR* as maximum release.

Effect of Lithium on Mouse Tumor Cell Growth *In Vivo*. Mouse melanoma cells (B16) were used as an experimental model. Each C57BL/6 mouse was inoculated intraperitoneally with 0.5 ml of 2×10 B16 cells. A total of 32 mice were then divided into four groups: (i) treatment with lithium alone, with injection of lithium chloride (2.5 mg/day) subcutaneously for 3 days before tumor cell transplantation; afterward, mice received the same dose of lithium every 1–2 days for a total of 11 times, (ii) treatment with IL-2/LAK, with, after inoculation, 3 days of IL-2 (1000 units/day) and 2 days of LAK cells ($1–2 \times 10^7$/day) injected alternatively into the mice for 5 days altogether; (iii) treatment with lithium in combination with IL-2/LAK; and (iv) control, with injection of 15% NCS RPMI 1640 only. The time course of tumor development, the size of tumor mass, and the survival of tumor-bearing mice were observed and recorded daily.

Effect of Lithium on TNF-α mRNA Expression of LAK Cells. LAK cells (1×10^7) with or without treatment of lithium were pelleted by centrifugation and then resuspended in 10 ml of cold phosphate-buffered saline. The cell pellets were dissolved with 1 ml of 4.2 *M* guanidine isothiocyanate. The total RNA was extracted according to the heat-phenol method described by Maniatis *et al.* (3). The resulting RNA pellet was washed once with 70% ethanol and dissolved in 100–150 μl of distilled water. The samples were denatured with formaldehyde and transferred onto nitrocellulose membrane using a slot blot apparatus. The nitrocellulose membranes were then baked at 80°C for 2 hr and kept in a vacuum until use.

The TNF-α cDNA fragment was labeled with [α-^{32}P]dCTP by the nick translation method. A specific activity was about 1×10^8 cpm/μg of cDNA. Hybridization was performed as described by Sambrook *et al.* (4). The nitrocellulose membrane was then exposed at −70°C to Kodak XAR-50 film using an intensifying screen.

Results

***In vitro* Effect of Lithium on Human and Mouse LAK Cell Activity.** PBMC were cultured with suboptimal IL-2 (40 units/ml) at 37°C in 5% CO_2 for 4 days for induction of LAK cells. In the presence of IL-2 and lithium, LAK cell activity was significantly enhanced as compared with control (Fig. 1). Where mouse splenocytes were used instead of PBMC, the splenocytes showed a similar enhancing effect (Fig. 2).

cAMP Involved in the Enhancing Effect of Lithium on LAK Activity. Cholera toxin is an adenylate cyclase activator that can increase cAMP levels (5). In order to determine whether the enhanced LAK cell activity caused by lithium was associated with cellular cAMP, we used CT. As shown in Figure 3, LAK activity was significantly inhibited by CT at a dose greater than

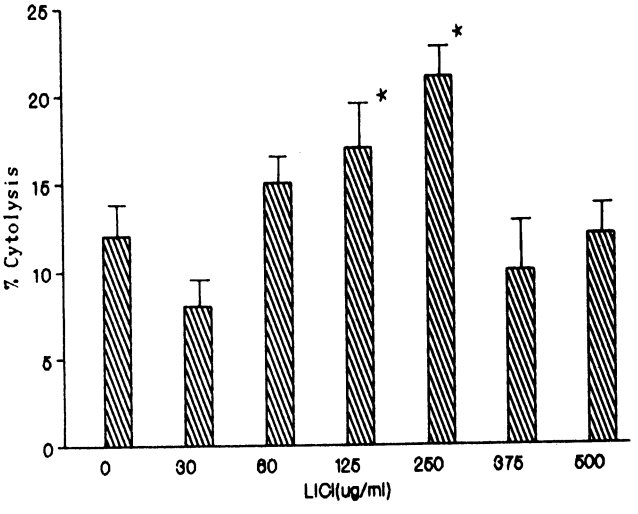

Figure 1. Effect of lithium concentrations on human LAK activity. PBMC (1×10^8/ml) were cultured with rIL-2 (40 units/ml) and various concentrations of lithium for 96 hr. Cells were then collected and assayed for LAK cell activity. *$P < 0.01$ compared with control.

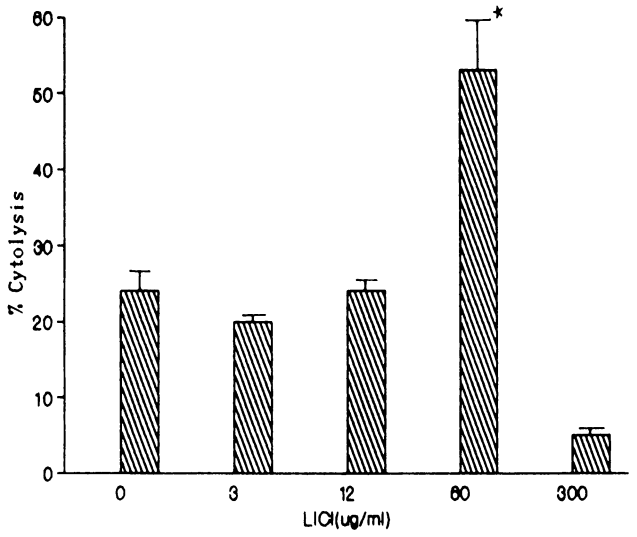

Figure 2. Effect of lithium concentrations on mouse LAK cell activity. Mouse splenocyte (5×10^8/ml) were cultured with rIL-2 (10 units/ml) and various concentrations of lithium for 96 hr. Cells were then collected and assayed for LAK cell activity. *$P < 0.05$ compared with control.

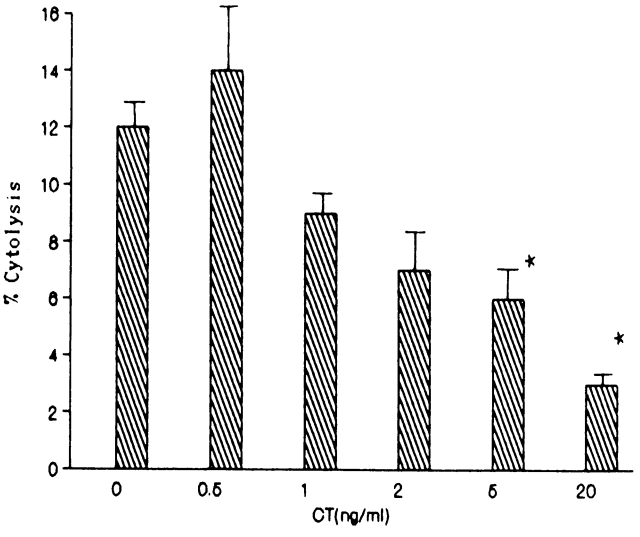

Figure 3. Effect of CT concentrations on human LAK cell activity. PBMC (1×10^8/ml) were cultured with rIL-2 (40 units/ml) and various concentrations of CT for 96 hr. Cells were then collected and assayed for LAK cell activity. *$P < 0.05$ compared with rIL-2 alone.

5 ng/ml ($P < 0.05$). However, as lithium was added in the presence of IL-2 and CT, LAK activity was partially restored (Fig. 4), which suggests that lithium might influence cAMP levels.

Effect of Lithium on TNF-α mRNA Induction in LAK Cells. It has been reported that TNF-α plays an important role in LAK cytotoxicity. The gene expression of TNF-α mRNA could be detected 3 days after LAK cell induction (6). This result was in accordance with our findings. Moreover, lithium could enhance TNF-α mRNA expression as compared with IL-2 treatment alone in LAK induction (Fig. 5).

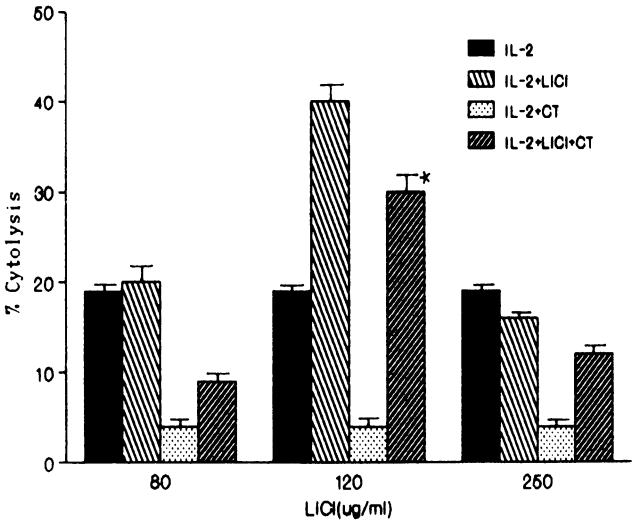

Figure 4. Effect of lithium on the inhibition of LAK cell activity by CT. PBMC were cultured with rIL-2 (40 units/ml), CT (5 ng/ml), and various concentrations of lithium for 96 hr. Cells were then collected and assayed for LAK cell activity. *$P < 0.05$ compared with treatment with rIL-2 in combination with CT.

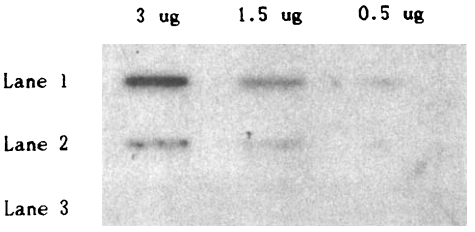

Figure 5. Enhancing effect of lithium on TNF-α mRNA in LAK cells. PBMC (1×10^8/ml) were cultured with 15% RPMI 1640 medium alone (Lane 3), 40 units/ml of rIL-2 (Lane 2) or 40 units/ml of rIL-2 and 125 μg/ml lithium (Lane 1) for 72 hr. Cells were then collected for extraction of total RNA and analysis of TNF-α mRNA.

Effect of Lithium on Antitumor Growth *In Vivo*. After B16 cell inoculation, the number of mice that developed palpable tumor nodules was six out of eight for control as compared with four out of eight for those treated with lithium alone, one of eight for those receiving IL-2/LAK, and zero of eight for those treated with IL-2/LAK/Li (Fig. 6). The days on which all transplanted mice developed tumor nodules were 11 (control), 13 (lithium alone), 14 (IL-2/LAK), and 15 (IL-2/LAK/Li), respectively. The time of tumor development in IL-2/LAK- and IL-2/LAK/Li-treated mice was longer as compared with the control mice ($P < 0.05$). However, there was no significant difference between IL-2/LAK and IL-2/LAK/Li treatment.

The mean survival time of tumor-bearing mice in these four groups was 23 days for control, 24.6 days for lithium alone, 23.6 days for the IL-2/LAK group, and 28.6 days for the IL-2/LAK/Li group. The mean survival time for the IL-2/LAK/Li group was statistically longer than that for the others ($P < 0.01$). However, there was no difference among the other three groups

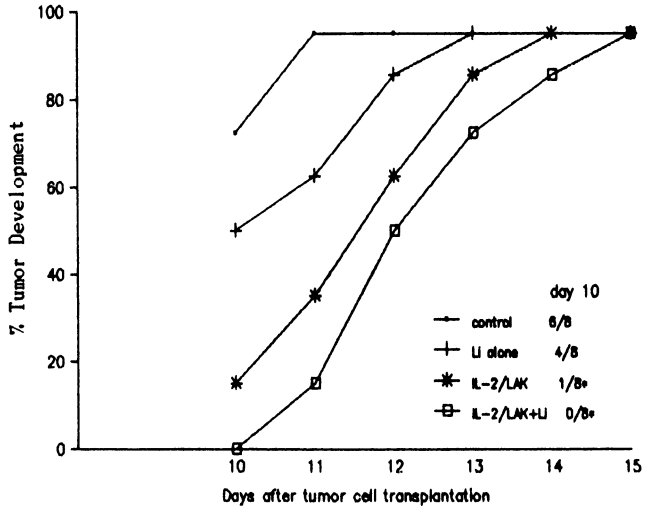

Figure 6. Effect of lithium on tumor development. After tumor cell transplantation, 32 mice were divided into four groups and treated by the method described previously. The time of tumor development in each group was recorded and the differences were calculated. *$P < 0.05$ compared with control.

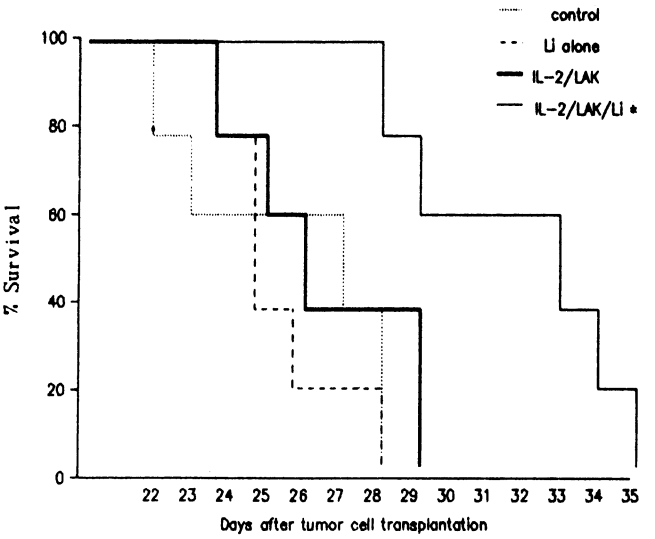

Figure 8. Effect of lithium on survival of tumor-bearing mice. Mice were treated as described previously. On Day 22 after tumor inoculation, the death of these mice occurred and the survival of mice was recorded daily. *$P < 0.05$ compared with rIL-2/LAK.

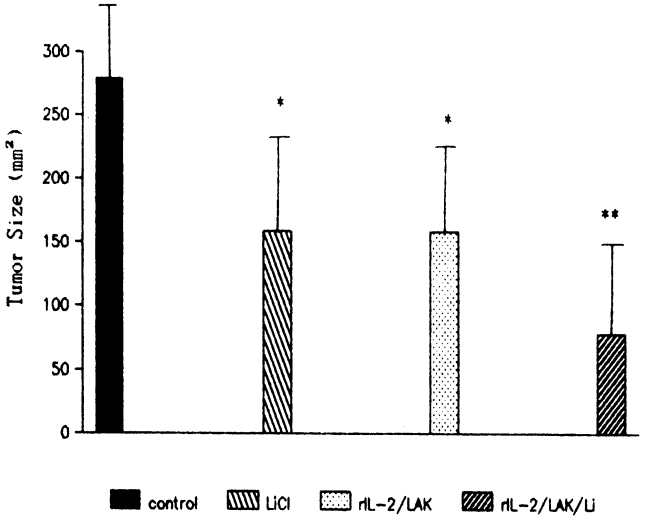

Figure 7. Inhibitory effect of lithium on tumor growth. Mice were treated as described previously. On Day 15, their tumor masses were measured and the differences were calculated. *$P < 0.01$ compared with control. **$P < 0.05$ compared with rIL-2/LAK.

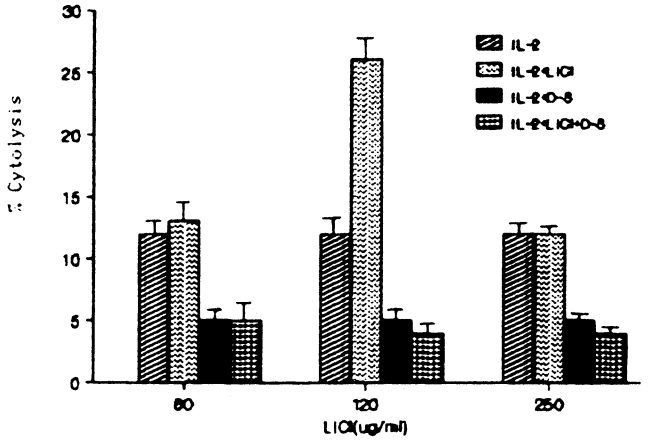

Figure 9. Effect of lithium on the inhibition of LAK cell activity by D-sphingosine (D-S). PBMC were cultured with rIL-2 (40 units/ml), D-sphingosine (30 μM), and various concentrations of lithium for 96 hr. Cells were then collected and assayed for LAK cell activity.

(Fig. 7). The size of the tumor mass was also observed. The tumor size in lithium alone and IL-2/LAK was smaller than that in control ($P < 0.05$), whereas the tumor size in IL-2/LAK/Li mice was the smallest, which was significantly different from IL-2/LAK mice (Fig. 8).

Discussion

Our previous studies indicated that lithium enhanced IL-2 production. Because IL-2 is a crucial cytokine in immune regulation and plays an important role in cancer immunotherapy, it is worthwhile to study further the relationship between lithium and LAK cell activity. Here, we first report the enhancing effect of lithium on human and mouse LAK cell activity. The *in vivo* study revealed that lithium exerted its antitumor effect in two aspects: (i) the tumor size of lithium in combination with IL-2/LAK was the smallest among the four groups; and (ii) lithium significantly prolonged the survival time of tumor-bearing mice. The mechanism of its antitumor action has not yet been elucidated. Lithium might directly enhance the LAK cell cytotoxic effect by secreting TNF-α or other cytotoxic factors. Lithium could also affect LAK cells indirectly by acting on T cells to produce IL-2 and interferon-γ (unpublished data). Both are important cytokines for activating antitumor effector cells such as natural killer cells and macrophages.

It has been reported that cAMP is a negative signal of LAK activity (7). Our results are in accordance with

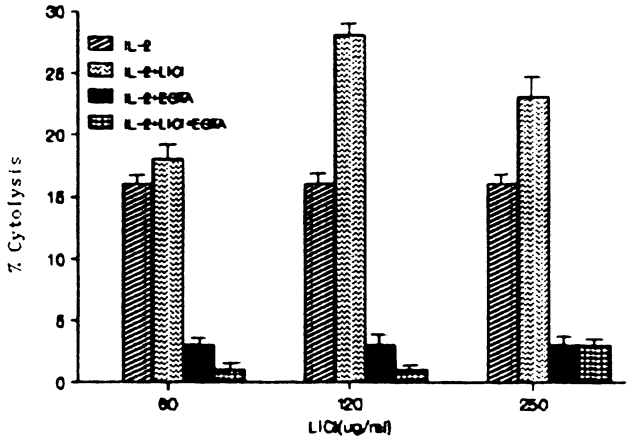

Figure 10. Effect of lithium on the inhibition of LAK cell activity by EGTA. PBMC were cultured with rIL-2 (40 units/ml). EGTA (0.5 μM), and various concentrations of lithium for 96 hr. Cells were then collected and assayed for LAK cell activity.

this finding. Furthermore, the inhibitory effect of LAK activity by CT could be partially reversed when lithium was added to the culture, which suggests that lithium is acting by decreasing cAMP levels. The protein kinase C inhibitor D-sphingosine and the calcium-chelating agent EGTA both showed an inhibitory effect on LAK cell activity. However, lithium could not reverse such effects (Figs. 9 and 10), which indicates that lithium affects LAK cell activity by directly regulating cyclic AMP levels in the adenylate cyclase system.

It has already been shown that immunotherapy with IL-2/LAK is useful for some cancer patients. However, there are two main problems to be solved: (i) the side effects of IL-2 and (ii) the tedious procedure and high cost. Lithium has been used in clinical treatment for patients with leukopoeic or dispyschiatric disease. The concentration of lithium in our study is relevant to the clinical dosage. According to these findings and our previous results, lithium affects several immune functions. Thus, it is promising that lithium can be used as an effective immunomodulator in immunotherapy of cancer and other immune diseases.

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