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Abstract

We investigated the effects of lymphokine-activated killer (LAK) cells on epidermal hyperplasia induced by cholera toxin (CT). LAK cells showed cytotoxic activity against both tumor cell lines and proliferating normal cells including skin epidermal cells. When 1 x 10(7) LAK cells were injected intradermally together with 1.0 ng of CT, epidermal hyperplasia was markedly suppressed. The LAK effectors inhibiting epidermal hyperplasia showed surface phenotypes of asialo-GM1+, Thy-1+, Lyt-2- and L3T4-, that were different from those of LAK cells killing tumor cells in vitro. Epidermal hyperplasia induced by CT was not suppressed by topical administration of cytokines such as interleukin-2, interferon and tumor necrosis factor. Therefore, the antiproliferative effect of LAK cells might be attributed to their direct action on the epidermal cells.

KEYWORDS: lymphokine-activated killer cell, cholera toxin, epidermal proliferation, cytokine

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We investigated the effects of lymphokine-activated killer (LAK) cells on epidermal hyperplasia induced by cholera toxin (CT). LAK cells showed cytotoxic activity against both tumor cell lines and proliferating normal cells including skin epidermal cells. When 1×10^7 LAK cells were injected intradermally together with 1.0 ng of CT, epidermal hyperplasia was markedly suppressed. The LAK effectors inhibiting epidermal hyperplasia showed surface phenotypes of asialo-GM1$^+$, Thy-1$^+$, Lyt-2$^-$ and L3T4$^-$, that were different from those of LAK cells killing tumor cells *in vitro*. Epidermal hyperplasia induced by CT was not suppressed by topical administration of cytokines such as interleukin-2, interferon and tumor necrosis factor. Therefore, the anti-proliferative effect of LAK cells might be attributed to their direct action on the epidermal cells.

Key words: lymphokine-activated killer cell, cholera toxin, epidermal proliferation, cytokine

The incubation of human peripheral blood lymphocytes or murine spleen cells with recombinant human interleukin-2 (rIL-2) generates lymphokine-activated killer (LAK) cells which are able to lyse a number of tumor target cells in ^{51}Cr release assays even when these tumors are resistant to natural killer (NK) cells (1-3). LAK cells can also lyse autologous lymphoblasts that have been modified with 2, 4, 6-trinitrobenzene sulfonic acid (4). LAK cells generated from spleen cells of hepatectomized mouse exhibit lytic activity against syngeneic lectin-induced lymphoblasts and regenerating liver cells (5, 6).

However, the role of LAK cells *in vivo* has not been thoroughly established. In tumor-bearing mice, intravenous adoptive transfer of LAK cells combined with systemic rIL-2 therapy can reduce the extent of liver and lung metastases. We have found that liver regeneration following partial hepatectomy is suppressed by intravenous administration of LAK cells (7). However, the characters of the effector cells which suppress liver regeneration could not be clarified since numerous lymphocytes were needed for the study. The effect of LAK cells on localized epidermal cell proliferation on mouse skin was studied in a small scale experimental model to elucidate the role of LAK cells *in vivo*.

Kuroki *et al.* (8-10) previously reported that cholera toxin (CT) stimulates *in vivo* and *in vitro* growth of epidermal cells. Intradermal injection of CT at doses of 0.2 ng or more into mice induces two successive synchronous divisions of epidermal basal cells, resulting in epidermal hyperplasia that reaches its peak on day 4 with the formation of 8 to 12 cell layers. The purpose of this paper is to demonstrate the effects of LAK cells on epidermal proliferation.

Materials and Methods

Animals. C3H/He male mice 6-10 weeks of age were obtained from Shizuoka Experimental Animal Farm (Hamamatsu, Japan). Mice were housed in groups of 10 or less per cage and fed an Oriental solid diet (Oriental Teast Co., Tokyo, Japan) in our experimental animal farm in compliance with animal care regulations.

Preparation of spleen cells. Mice were killed by cervical dislocation, and their spleens were harvested aseptically, minced with scissors, and passed through a 150 plantinum mesh. The cells were centrifuged at 1,500 rpm for 5 min and the pellet was resuspended in 0.85 % ammoninum chloride buffer to lyse contaminating erythrocytes. One min later, the suspension was diluted in culture medium and washed three times. The final cell pellet was then suspended in a complete medium (CM).

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The CM was composed of RPMI 1640 (Nissui Pharmaceutical Co., Tokyo Japan) supplemented with 25 mM N'-2 hydroxyethyl-piperazine N'-2-ethane sulfonic acid (HEPES) buffer (Sigma Chemical Co., St. Louis, MO, USA), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 5×10^{-5} M 2-mercaptoethanol, and 10 % heat-inactivated fetal calf serum (FCS; Gibco Laboratories, NY, USA).

LAK cell generation. Spleen cell suspensions were prepared at a concentration of 2.5×10^6 cells/ml, and incubated in the presence of rIL-2 (200 U/ml; Shionogi & Co., Ltd., Osaka, Japan) in CM at 37°C in 5 % CO_2 for 4 days. Cells were cultured in 250-ml flasks (Falcon 2034). The culture supernatant of LAK cells was harvested on day 4 and examined for unknown soluble factors secreted by LAK cells.

Tumor cell lines. JTC-11 cells derived from Ehrlich carcinoma and EL-4 leukemia cells were used as targets in cytotoxicity assay.

Preparation of skin epidermal cells. The trypsinization method of Steinmuller and Wunderlich (11) was used to prepare skin epidermal cells (SEC). Murine tail skin was cleaned with 70 % ethanol, removed entirely with a scalpel, and cut into small pieces. The pieces were then floated, with their dermal side down, on 0.5 % trypsin-phosphate-buffered saline (PBS) (Gibco). The pieces were soaked in 0.025 % DNase (Sigma) after incubation at 37°C for 2 h. The superficial layer of the epidermis was removed by forceps, and the exposed basal layer was rubbed with a heat-polished glass bar to free the epidermal cells. Isolated skin cells were filtered through a 150 platinum mesh to remove debris and hair, washed three times with PBS, and suspended in CM.

Antibody and complement treatment. LAK cells (1×10^7/ml) were incubated with antibodies at the appropriate dilutions at 4°C for 1 h with intermittent agitation. Anti-asialo-GM1 (AGM1) (Wako Pure Chemical Indusries Ltd., Osaka, Japan), anti-Thy-1.2, anti-Lyt-1.1, anti-Lyt-2.1 (New England Nuclear, Boston, MA, USA), and anti-L3T4 (Becton-Dickinson, Mountain View, CA, USA) were used at dilution of 1:100, 1:50, 1:200, 1:100 and 1:100, respecively. The cells were washed once, resuspended at 1×10^7/ml in a 1/10 dilution of low-toxicity rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada), incubated at 37°C for 45 min, then washed three times, and finally resuspended in CM for further study.

^{51}Cr release cytotoxicity assay. LAK activity was assessed by a ^{51}Cr release assay performed in triplicate. Various numbers of effector cells were incubated with 1×10^4 $Na_2{}^{51}CrO_4$ (New England Nuclear)-labeled target cells in a total CM volume of 0.2 ml per well in 96-well microtiter plates. The plates were incubated at 37°C in 5 % CO_2 for 4 h or 12 h, and then 0.1 ml of the supernatant from each well was carefully harvested with a micropipette and counted for 2 min in the Aloka ARC-451 gamma counter. Spontaneous release was determined by culturing ^{51}Cr-labeled target cells alone, and total labeling was determined by counting half of the plated target cells. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100$$

Administration of cholera toxin. CT was obtained from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). A stock solution was prepared by dissolving the CT at 1 mg/ml in 0.05 % Tris buffer (pH 8.0) containing lactose (50 mg/ml) as a stabilizer. This solution was diluted with cold PBS and 0.1-ml aliquots were injected intradermally into the backs of mice. Four days later, the injected skin site was removed, fixed in 10 % formalin, sectioned after embedding in paraffin, and stained with hematoxylin and eosin. The thickness of the epidermis was measured on a photomicrograph. Each experimental group consisted of at least three mice. Statistical significance of the differences in epidermal thickness among the groups was analysed using Student's unpaired t-test.

Cytokines. Natural human tumor necrosis factor (TNF)-α, TNF-β, natural murine interferon (IFN)-α/β, and recombinant murine IFN-γ were supplied by Hayashibara Biochemical Laboratories Inc. (Okayama, Japan).

Results

LAK activity. Lymphocytes from C3H/He mice cultured with 200 U/ml of rIL-2 for 4 days demonstrated strong lytic activity against NK-resistant tumor cells, JTC-11 and EL-4 (Tables 1 and 2). Effector cells were depleted of the AGM1$^+$, Thy-1$^+$, Lyt-1$^+$, Lyt-2$^+$ and L3T4$^+$ subsets using antibodies and complement to determine the phenotype of the cells responsible for LAK activity. Treatment with anti-AGM1, anti-Thy-1.2 or anti-Lyt-2.1 antibody significantly reduced the LAK

activity against JTC-11 cells. while anti-Lyt-1.1 or anti-L3T4 antibody had no effect (Table 1). More interestingly, LAK cells exhibited greater significant cytotoxic activity against epidermal cells than spleen cells not treated with rIL-2 (Table 2).

Epidermal hyperplasia induced by CT. Injection of CT into the dorsal skin of C3H/He mice produced marked epidermal hyperplasia at doses of 1-100 ng (data not shown). The thickness of the epidermis increased progressively, and was greatest on day 4, and decreased to the normal level on day 7. The epidermis 4 days after the injection of 1.0 ng of CT was 4-fold thicker than that in normal skin. However, when 1×10^7 LAK cells were injected with the same dose of CT, epidermal hyperplasia was markedly suppressed on day 4 (Figs. 1 and 2). In contrast, intradermal injection of fresh spleen cells had no effect on CT-induced epidermal hyperplasia (Fig. 1).

Table 1 Lymphokine-activated killer (LAK) activity and surface phenotypes of LAK effector cells

Effector[a]	Treatment	% cytotoxicity[b] (E:T = 40:1)
LAK	Untreated	47.8 ± 3.2
	Complement (C)	51.1 ± 4.3
	C + α AGM1	25.9 ± 3.5
	C + α Thy-1.2	25.0 ± 3.1
	C + α Lyt-1.1	51.5 ± 4.7
	C + α Lyt-2.1	39.9 ± 4.2
	C + α L3T4	52.7 ± 4.3
SPC	Untreated	2.8 ± 0.7

(Significance brackets: Complement vs C + α AGM1, c; Complement vs C + α Thy-1.2, c; Complement vs C + α Lyt-2.1, d.)

a: LAK; spleen cells isolated from C3H/He mice and cultured with 200 U/ml of recombinant interleukin (rIL)-2 for 4 days. SPC; mouse spleen cells not treated by rIL-2

b: Cytotoxicity was determined in a 12 h ^{51}Cr release assay using JTC-11 as target cells. The results are representative data of three independent experiments. E: effector, T: target.

c: Significant difference; $p < 0.01$.

d: Significant difference; $p < 0.05$.

AGM1: asialo GM1.

Table 2 Percent cytotoxicity of various effectors for skin epidermal cells (SEC)

Effector (E)	Target (T)	E : T 200:1	100:1	50:1	25:1	12.5:1
SPC	EL-4	0.8	1.6	2.2	1.0	—
LAK[a]	EL-4	—	47.8	36.2	27.8	18.6
SPC	SEC[b]	0.4	0.4	1.2	0.8	—
LAK	SEC	—	20.4	16.2	16.0	11.4

a: Murine spleen cells were cultured with 200 U/ml of rIL-2 for 4 days.

b: Skin epidermal cells were isolated from mouse tail skin by the method of Steinmüller and Wunderlich.

LAK, SPC, rIL-2: See Table 1.

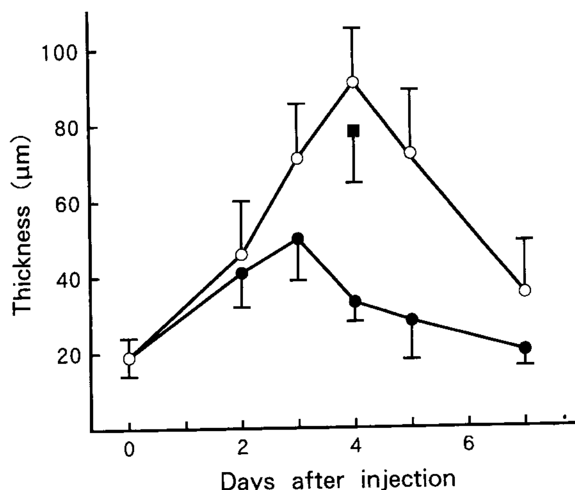

Fig. 1 Time course of epidermal hyperplasia induced by cholera toxin (CT). C3H/He mice were injected with 1.0 ng of CT alone (○), 1.0 ng of CT plus 1×10^7 lymphokine-activated killer (LAK) cells (●) or 1.0 ng of CT plus 1×10^7 fresh spleen cells (■). The epidermal thickness was then mesured on photomicrographs.

Phenotypes of LAK cells that suppressed epidermal hyperplasia. LAK cells were pre-treated with various antibodies plus complement and then injected intradermally immediately after CT injection to determine surface phenotypes of the effector cells which suppressed epidermal hyperplasia in the mice. LAK cells treated with anti-Lyt-1, anti-Lyt-2 or anti-L3T4 antibody still suppressed epidermal hyperplasia to the same extent as untreated LAK cells, but LAK cells treated with anti-AGM1 or anti-Thy-1.1 antibody lost most of their suppresive activites. The loss indicated that the *in vivo* effector cells were $AGM1^+$, $Thy\text{-}1^+$ and $Lyt\text{-}2^-$ lymphocytes (Table 3).

Cytokines. Culture supernatant of LAK cells had no effect on epidermal hyperplasia. We investigated whether cytokines, which had already been purified,

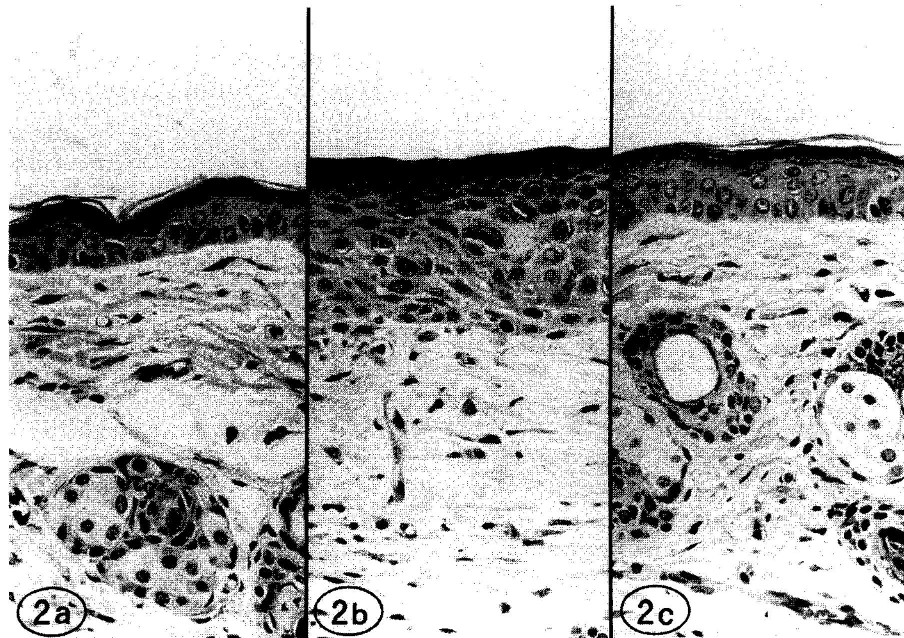

Fig. 2 Microscopic appearance of epidermal hyperplasia induced by CT. The epidermis 4 days after the injection of 1.0 ng of CT (2b) was 4-fold thicker than that in normal skin (2a). When 1×10^7 LAK cells were injected with 1.0 ng of CT, epidermal hyperplasia was remarkably suppressed on day 4 (2c). CT, LAK: See Fig. 1.

Table 3 Phenotype of lymphokine-activated killer (LAK) effector cells which suppressed cholera toxin (CT)-induced epidermal hyperplasia

Treatment of LAK cells[a]	n	Eepidermal thickness[b] (μm; mean $\pm$ SD)
—	3	86.1 $\pm$ 15.2
Untreated	3	41.8 $\pm$ 6.3
C + αAGM1	3	80.7 $\pm$ 7.5[c]
C + αThy-1.2	3	70.8 $\pm$ 13.4[d]
C + αLyt-1.1	3	50.2 $\pm$ 7.8
C + αLyt-2.1	3	52.9 $\pm$ 13.3
C + αL3T4	3	46.2 $\pm$ 6.6

a: LAK cells (1×10^7), either untreated or treated with the indicated antibody and complement, were injected simultaneously with 1.0 ng of CT.
b: Four days after injection, the epidermal thickness was measured on photomicrographs. The results are reperesentative data of two separate experiments.
c: Significant difference from the group given CT and untreated LAK cells; $p < 0.01$.
d: Significant difference from the group given CT and untreated LAK cells; $p < 0.05$.
AGM1; See Table 1.

Table 4 Effects of various cytokines on cholera toxin (CT)-induced epidermal hyperplasia

Cytokine[a]	Dose(U)	n	Thickness(μm)[b]
—		3	93.3 $\pm$ 12.4
LAK sup[c]		3	87.5 $\pm$ 12.3[d]
TNF-α	10^5	3	80.9 $\pm$ 17.2[d]
TNF-α	10^4	3	83.4 $\pm$ 5.5[d]
TNF-β	10^5	3	93.1 $\pm$ 18.2[d]
TNF-β	10^4	3	76.3 $\pm$ 16.9[d]
IL-2	10^5	3	79.5 $\pm$ 18.0[d]
IL-2	10^4	3	80.8 $\pm$ 5.2[d]
IFN-α/β	10^3	3	75.4 $\pm$ 15.3[d]
IFN-α/β	10^2	3	77.9 $\pm$ 13.8[d]
IFN-γ	10^3	3	78.2 $\pm$ 11.2[d]
IFN-γ	10^2	3	82.0 $\pm$ 7.2[d]

a: The indicated doses of natural human tumor necrosis factor (TNF) -α and TNF-β, recombinant human interluekin (IL)-2, natural murine interferon (IFN)-α/β, and recombinant murine IFN-γ were injected simultaneously with 1.0 ng of CT.
b: Four days after injection, the epidermal thickness was measured on photomicrographs. The results are representative data of two separate experiments.
c: Culture supernatants of LAK cells were injected simultaneously with 1.0 ng of CT.
d: Not significantly different from the group given CT alone.

could suppress the hyperplasia. Table 4 indicates that IL-2, IFNs and TNFs had no effect on CT-induced epidermal hyperplasia.

Discussion

LAK cells have been used in adoptive immunotherapy for cancer owing to their ability to destroy a broad spectrum of target cells (1-3). Initial studies indicated that LAK cells were only cytotoxic to tumor cells and not to normal cells. Howevemr, the lytic activity expressed by LAK cells is in fact not limited to tumor cells because they can also lyse autologous lymphoblasts modified with 2, 4, 6-trinitrobenzene sulfonic acid (4). We have previously demonstrated that spleen cells from normal mice exhibit augmented NK and LAK activity following partial hepatectomy (5, 6). Furthermore, LAK cells generated from hepatectomized mice are cytotoxic to lectin-stimulated lymphoblasts and regenerating liver cells. These results suggest that the immune system may not only distinguish between self and not-self, but may also regulate the proliferation of autologous normal cells and tissues. A previous study provided some *in vivo* evidence supporting this hypothesis. When a large number of LAK cells were injected intravenously immediately after partial hepatectomy, liver regeneration at 36 h was significantly suppressed as shown by ^{3}H-thymidine uptake or BrdU labeling of hepatocytes (12). However, it remains unclear whether LAK cells can suppress the recovery of liver weight. There is an experimental limitation in this study because a large number of lymphocytes would be necessary. In this study, we examined the suppressive effect of LAK cells on localized epidermal proliferation on mouse skin, since a relatively small number of lymphocytes would be enough for the experiment.

Initially, the characteristics of LAK cells showing cytotoxic activity against tumor cells *in vitro* was analysed. The cytotoxic activity against JTC-11 cells which were resistant to NK cells was partially eliminated by anti-AGM1, anti-Thy-1 and anti-Lyt-2 antibodies, respectively. LAK cells also demonstrated cytotoxic activity against epidermal cells prepared from murine tail skin, although the activity was slight, and the surface markers of killer cells remain to be investigated.

Secondly, the *in vivo* effect of LAK cells was assessed in epidermal hyperplasia induced by CT. CT-induced epidermal hyperplasia was significantly suppressed by a large number of LAK cells injected into the epidermis. These results suggest that LAK cells may take part in the surveillance of tumor cells, and regulate the proliferation of non-tumor cells such as hepatocytes and skin epidermal cells. The lymphocytes that suppressed epidermal proliferation were AGM1$^+$, Thy-1$^+$ and Lyt-2$^-$ cells. Although LAK cells generated *in vitro* are known to be AGM1$^-$, Thy-1$^+$ and Lyt-2$^+$, LAK-like cells generated *in vivo* by administration of IL-2 are reported to be present in the population of AGM1$^+$ as well as Thy-1$^+$ lymphocytes (13-15). Activated lymphocytes bearing AGM1 may play a part in the regulatory system of proliferation of normal cells.

The mechanism of the cytostatic/cytotoxic activity of LAK cells remains unclear. Gangi has demonstrated that LAK cells produce cytotoxic factors against L929 cells and that TNF is one of these factors (16). IFNs have been known to regulate cell proliferation and differentiation (17), and IFN-α/β has been reported to inhibit mouse liver regeneration after partial hepatectomy (18). In this study, we demonstrated that culture supernatant of LAK cells and purified cytokines (IL-2, IFN-α/β, IFN-γ, TNF-α, and TNF-β) had no suppressive effect of CT-induced epidermal hyperplasia. Transforming growth factor-β (TGF-β) has been shown to be a potent growth inhibitor for many cell types, such as epithelial cells (19), keratinocytes (20), fibroblasts, B and T lymphocytes (21), and normal diploid hepatocytes (22). TGF-β may be involved in hepatocyte growth response *in vivo*, because increase of mRNA of TGF-β is observed in the non-parenchymal cells of the regenerating liver, reaching a peak after a major wave of hepatocyte cell division. Because proliferating lymphocytes are known to have increased TGF-β mRNA (24), it is possible that the LAK cells injected in the skin may produce TGF-β *in vivo*, which results in the inhibition of epithelial hyperplasia.

Another possibility may be deduced from this study. It has been known that keratinocytes of epidermis produce a cytokine which stimulates proliferation of thymocytes, and is called epidermal cell-derived thymocyte-activating factor (ETAF) (25). Recently, ETAF has been proved to be identical to interleukin-1 (IL-1) (26). IL-1 acts as a growth factor for keratinocytes as well as a chemoattractant for leukocytes. It is usually fixed on the cell surface, acting as an autocrine growth factor (27). But in the inflammatory condition, IL-1 is produced abundantly, and released into the surrounding tissue as a

chemotactic factor (28). In this experiment, locally injected LAK cells might be attracted to the region near the CT-injected epidermal cells by IL-1 that the proliferating keratinocytes secret. LAK cells might be tightly conjugated with the keratinocytes by the action of other cytokines produced during the local accumulation. The lymphocyte population including LAK cells is known to produce IFN-γ (29). IFN-γ increases the adherence of lymphocytes to keratinocytes (30), so the adhesion of LAK cells with keratinocytes might also be enhanced. With the aid of these cytokines to enhance keratinocyte-lymphocyte interaction, LAK cells that exhibit weak cytotoxic activity against epidermal cells *in vitro* may suppress epidermal proliferation *in vivo* by their killing activity. It was reported that patients who underwent immunotherapy with IL-2 and LAK cells developed an eruption characterized histologically by infiltration of T lymphocytes and focal vacuolar degeneration of the basal cells (31). However, no pathological changes of the skin were observed in this study. Therefore, the antiproliferative action of LAK cells on epidermal cells is considered to be a different phenomenon from the dermatologic changes associated with IL-2 administration.

Further study is needed to elucidate the precise mechanism of anti-proliferative action of LAK cells.

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