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Augmentation of Cytotoxic Activity by Combination with Interleukin 2 and Interferon γ

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Summary

The synergy of cytotoxic activity by interleukin 2 (IL-2) and interferon γ (IFN-γ) was evaluated in human peripheral blood mononuclear cells (PBMC) and spleen cells. PBMC incubated with IL-2 (10 IU/ml) and IFN-γ (200 IU/ml) for 4 days showed the stronger cytotoxic activity against K562, MOLT-4 and Daudi cells. Combination with IL-2 and IFN-γ induced stronger activity than IL-2 or IFN-γ alone. In order to investigate the sequential roles of IL-2 and IFN-γ in the killer cell function, the cells were stimulated with IFN-γ after washing of IL-2 or stimulated by IFN-γ at various timing and duration without washing of IL-2. IL-2 was essential to induce the synergistic effect of IL-2 and IFN-γ to cytotoxic activity. The similar augmentation of cytotoxic activity was observed by the addition of IFN-γ at any incubation periods with IL-2, compared with stimulation with IL-2 or IFN-γ alone. The phenotypes of the killer cells by stimulation with IL-2, IFN-γ alone or IL-2 plus IFN-γ were mainly $CD2^+$, $CD16^+$, indicating the activated natural killer cells.

Introduction

We have recently reported that lymphokine-activated killer (LAK) activity of murine spleen cells was augmented by the combination with interleukin 2 (IL-2) and interferon γ (IFN-γ) in our laboratory[19]. However, in human model the effects of IFN-γ for LAK activity has not been fully known, and moreover the mechanism of the augmentation by the combination of IL-2 and IFN-γ has not been clarified in detail yet.

The present study was designed to clarify the role of IFN-γ to LAK activity and the phenotype of the effector cells by the combination of IL-2 and IFN-γ in peripheral blood mononuclear cells (PBMC) of healthy volunteers or cancer patients, and spleen cells of patients with gastric cancer.

Materials and Methods

1. Culture media

Recombinant human IL-2 (Shionogi Pharmaceutical Co., Japan) and recombinant human

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IFN-γ (Kyowa Hakko Co., Japan) were used for cell-activation. PRMI 1640 medium (GIBCO, Grand Island, New York, USA) was supplemented with 10% heat-inactivated human AB serum obtained from healthy subjects, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mmol/L glutamine (complete medium).

2. Established tumor cell lines

K562 was used as a NK-sensitive tumor cell line established from human erythroleukemia cells. MOLT-4 was used as a NK-sensitive tumor cell line established from human leukemia. Daudi was used as a NK-resistant tumor cell line established from human Burkitt's lymphoma.

3. Induction of effector cells

PBMC from 18 patients with gastrointestinal cancer and PBMC from 17 healthy subjects (who agreed with this research) were collected by layering onto Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuging at 400 g for 30 min at 20°C as previously described[17]. Spleens were resected from 8 gastric cancer patients aseptically. Spleens were minced on plastic dish (Corning No. 3003) with RPMI 1640, then passed through stainless mesh, and the spleen cells were separated by Ficoll-Hypaque gradients. The cells incubated with 10 IU/ml of IL-2 and/or various concentrations of IFN-γ at 37°C in a humidified 5% CO_2 atmosphere for 4 or 7 days in 24 well microtiter plates (Corning No. 25820).

4. Cytotoxic assay

A 4-h ^{51}Cr-release assay was performed. Target cells were labeled with 100 μ Ci of $Na_2{}^{51}CrO_4$, and washed three times. The ^{51}Cr-labeled target cells (100 μl; 1×10^5/ml) were then cultured in triplicated with 100 μl aliquots of effector cell suspensions (the effector to target ratio was fixed at 15 : 1) in round-bottomed microtiter plates (Corning No. 25850). After a 4-h incubation at 37°C, the radioactivity of the supernatants was determined using a gamma counter. Spontaneous release did not exceed 10% for K562 cells, 15% for Daudi cells and MOLT-4 cells of maximum release that was obtained by 1 N HCL. The percentage cytotoxicity was calculated as follows (all ^{51}Cr values in cpm).

$$\frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\,(\%)$$

5. Phenotypic analysis

The phenotypic analysis was performed by negative selection method as previously described[18]. The effector cells were suspended at 1×10^7/ml, to which was added 100 μl of monoclonal antibodies/ml for 60 min at 4°C. Then, after washed twice effector cells were treated by complement (C′, low-tox-H Rabbit Complement; Cedarlane Laboratories, Ontario, Canada). Using these effector cells, a 4-h ^{51}Cr-release assay was performed, as previously described[18]. The percent inhibition was measured as follows;

$$\left(1-\frac{C'+Ab}{C' \text{ alone}}\right)\times 100\,(\%)$$

Results

1. The concentration and culture periods of IL-2 and IFN-γ

The cytotoxic activity of the effector cells incubated with IL-2 alone was measured against K562 (Fig. 1). Over 100 IU/ml of IL-2, the cytotoxic activity reached its maximum and became plateau. The cytotoxic activity induced IFN-γ alone increased as a dose dependent manner (Fig. 2). From these results, 10 IU/ml of IL-2, the suboptimal concentration, and 200 IU/ml of IFN-γ were used in all subse-

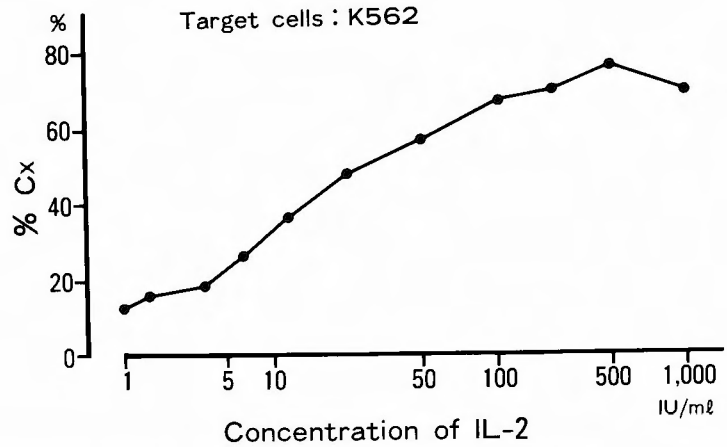

Fig. 1 The cytotoxic activity against K562 reached the plateau over 100 IU/ml of IL-2. In order to investigate the effect of the combination with IL-2 and IFN-γ, 10 IU/ml of IL-2 was used as the suboptimal concentration.

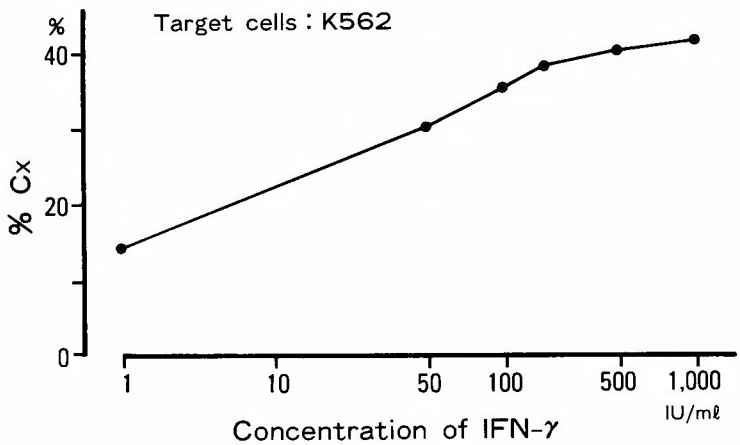

Fig. 2 The cytotoxic activity against K562 increased as a dose dependent manner by IFN-γ. 200 IU/ml of IFN-γ was used in all subsequent investigations.

quent investigations in order to examine the effect of IL-2 plus IFN-γ. The cytotoxic activity for 4 days culture incubated with IL-2 plus IFN-γ showed maximum rather than for 2 or 3 days culture (Fig. 3). Therefore, the culture period was determined on 4 days.

2. The augmentation of the cytotoxic activity by the combination with IL-2 plus IFN-γ

The augmentation of the cytotoxic activity against K562 by the combination with IL-2 plus IFN-γ was indicated in Fig. 4. In PBMC of healthy subjects, the cytotoxic activity by IL-2 alone was 60% and IFN-γ alone was 40%. The cytotoxic activity cultured with IL-2 and IFN-γ augmented to 75% ($p<0.01$). The cytotoxic activity in PBMC of cancer patients was lower than that of healthy subjects. However, the activity incubated with IL-2 plus IFN-γ (43%) was higher than that of IL-2 alone (30%) or IFN-γ alone (25%, $p<0.01$). Moreover, in spleen cells the similar augmentation by IL-2 plus IFN-γ was recognized ($p<0.01$). Fig. 5 showed the cytotoxic activity incubated with IL-2 and IFN-γ against MOLT-4 and Daudi cells. The similar results in the effects of IL-2 plus IFN-γ were obtained.

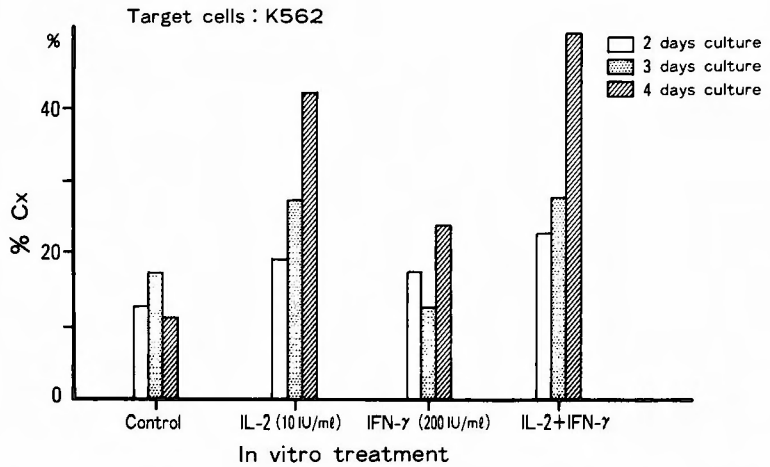

Fig. 3 To determine the optimal incubation periods, the cytotoxic activity of the effector cells incubated with IL-2 and/or IFN-γ was measured against K562. For 4 days culture ( ) the cytotoxic activity was augmented higher than that for 2 days culture ( ) or 3 days culture ( ).

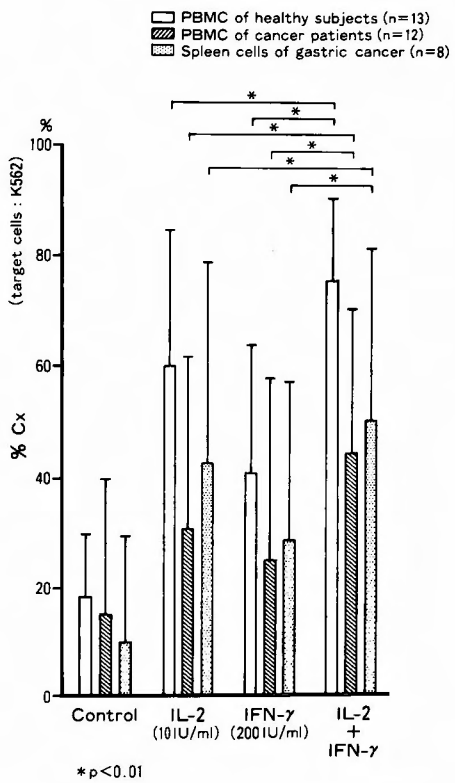

Fig. 4 In PBMC of healthy subjects ( ) and cancer patients ( ), and spleen cells of gastric cancer ( ), the cytotoxic activity against K562 was augmented by the combination with IL-2 plus IFN-γ, as compared with IL-2 alone or IFN-γ alone ($p<0.01$).

3. The sequential role of the combination of IL-2 and IFN-γ

In order to clarify the sequential role of IL-2 and IFN-γ in the augmentation of the cytotoxic activity, the sequence and incubation period of IL-2 and IFN-γ were altered diversely. After washing of IL-2 at

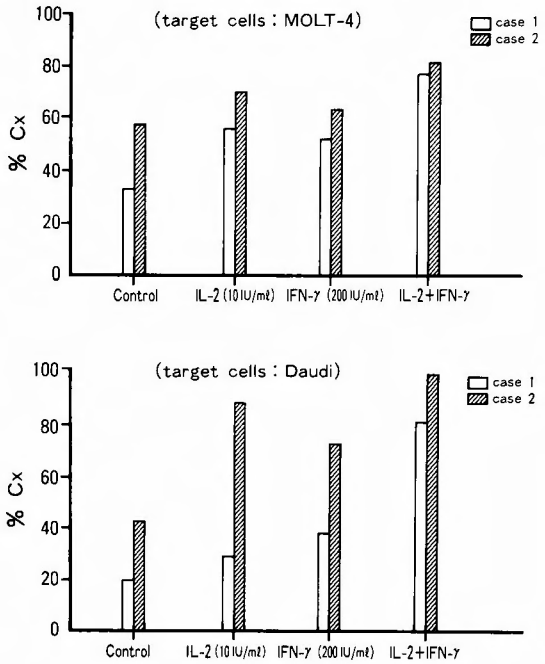

Fig. 5 In the case of MOLT-4 (above) or Daudi (below) as target cells, the cytotoxic activity was augmented by the combination with IL-2 plus IFN-γ, as compared with IL-2 alone or IFN-γ alone.

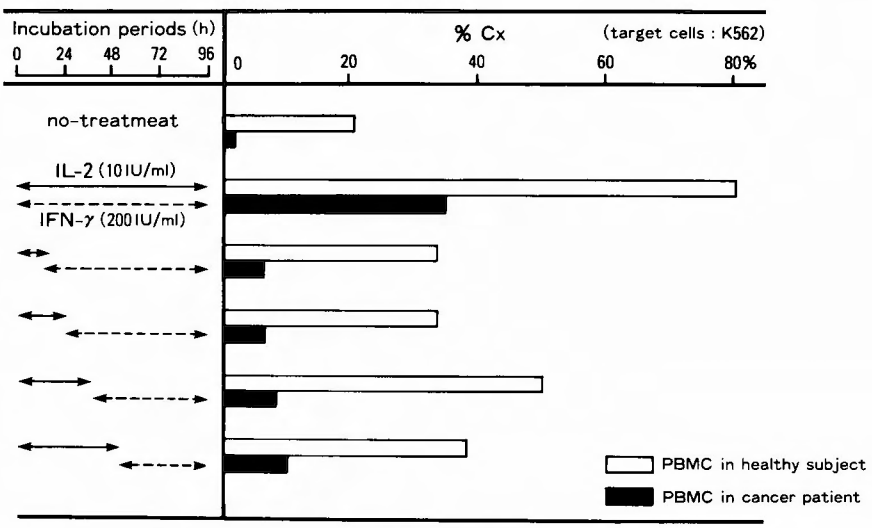

Fig. 6 The role of IL-2 and IFN-γ was investigated. With washing of IL-2 at 12, 24, 36 and 48 hours from the beginning of the culture, the cytotoxic activity was inhibited as compared with the combination with IL-2 plus IFN-γ from the beginning of the culture. Therefore, IL-2 was essential at any incubation period.

12, 24 and 36 hours from the beginning of the culture and then IFN-γ was added. The cytotoxic activity was inhibited significantly as compared with that cultured with IL-2 and IFN-γ in all incubation period (Fig. 6). Thus it was clarified that IL-2 was essential to the augmentation of cytotoxic activity in any incubation periods. Next, under the existence of IL-2, the timing and duration in addition to IFN-γ were altered variously and then the cytotoxic activity was measured. The alteration of the timing and duration of IFN-γ did not affect the cytotoxic activity (Fig. 7).

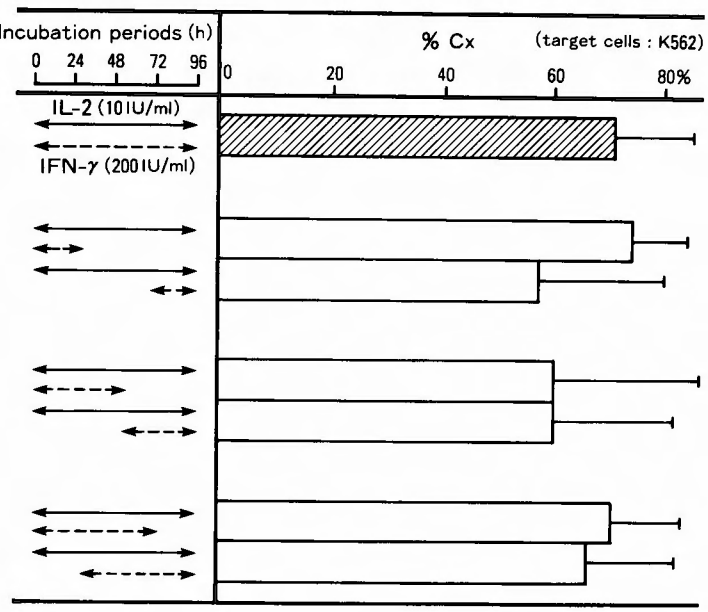

Fig. 7 Without washing of IL-2, the variations of the timing and duration on stimulating with IFN-γ did not affect the cytotoxic activity.

Table 1 The phenotypic analysis in PBMC of healthy subjects and cancer patients

(target cells:K562)

In vitro treatment		% Reduction		
		IL-2 (10 IU/mℓ)	IFN-γ (200 IU/mℓ)	IL-2+IFN-γ
C'+anti-CD2	healthy subjects	30±9	43±6	41±8
	cancer patients	31±9	28±7	36±7
C'+anti-CD3	healthy subjects	0	0	21
	cancer patients	1±0	5±1	5±2
C'+anti-CD8	healthy subjects	13±7	10±6	19±8
	cancer patients	16±6	5±2	6±3
C'+anti-CD16	healthy subjects	71±8	80±7	58±8
	cancer patients	76±10	72±8	53±8
C'+anti-Leu7	healthy subjects	45±12	50±12	45±8
	cancer patients	38±12	38±9	30±9

The phenotypic analysis in PBMC of healthy subjects and cancer patients was measured by negative selection method.

Table 2 The phenotypic analysis of spleen cells in patients with gastric cancer

(target cells:K562)

In vitro treatment	% Reduction		
	IL-2 (10 IU/mℓ)	IFN-γ (200 IU/mℓ)	IL-2+IFN-γ
C'+anti+CD2	19±10	23±9	20±9
C'+anti-CD3	12±12	15±8	11±10
C'+anti-CD8	12±8	11±5	10±6
C'+anti-CD16	69±10	33±7	26±12
C'+anti-Leu7	20±18	26±9	19±9

In spleen cells of gastric cancer patients incubated with IL-2 and/or IFN-γ, the treatment of anti-CD2 Ab or anti-CD16 Ab inhibited the cytotoxic activity.

4. The phenotype of the effector cells

Table 1 showed the phenotypes of the effector cells from PBMC of healthy subjects and cancer patients incubated with IL-2 and/or IFN-γ by negative selection method. In PBMC of healthy subjects and cancer patients incubated with IL-2 or IFN-γ alone, the treatment by anti-CD2 Ab inhibited the cytotoxic activity and similary anti-CD16 Ab and anti-Leu7 Ab strongly inhibited. However, the treatment with anti-CD3 Ab never inhibited and anti-CD8 Ab slightly inhibited the cytotoxic activity. In these effector cells incubated with IL-2 plus IFN-γ, the treatment with anti-CD2 Ab, anti-CD16 Ab and anti-Leu7 Ab strongly inhibited the cytotoxic activity, and in cancer patients, the treatment with anti-CD3 Ab and anti-CD8 Ab did not inhibit the cytotoxic activity compared to healthy subjects. Table 2 showed the phenotypic analysis of the effector cells from spleen cells of patients with gastric cancer. In spleen cells incubated IL-2 alone, the treatment by anti-CD2 Ab inhibited 19% of the cytotoxic activity, and anti-CD16 Ab did 69%, and anti-Leu7 Ab did 20%, respectively. However, the treatment by anti-CD3 Ab and anti-CD8 Ab slightly inhibited only 12% each other. In effector cells incubated IFN-γ alone and IL-2 plus IFN-γ, the similar patterns were observed.

Discussion

It has been demonstrated that PBMC from cancer patients incubated with IL-2 in vitro induces the killer cells which had non-specific tumor killing activity and these killer cells is designated as "LAK" cells[12]. The clinical trials were performed using LAK cells as the effector of adoptive immunotherapy. However, the various side effects including edema, dyspnea, liver, renal dysfunction and fever occurred due to the high doses of IL-2. Moreover, the clinical efficacy by adoptive immunotherapy using LAK cells has not been adequate yet. In the present study, we investigated whether the combination of IFN-γ and IL-2 augments the cytotoxic activity of effector cells.

There are numerous various reports that IL-2 and IFN-γ acted the augmentation of killer activity of PBMC[1,5]. NK activity of murine spleen cells was augmented by the incubation with IFN or poly I: C, a IFN inducer, and NK cell itself produced IFN and then the activity augmented by autocrine system. Therefore, it is called "IFN-NK system"[1,2]. NK cells proliferated by IFN, mainly IFN-γ from the effector

cells stimulated by IL-2 dependently[5]. On the other hand, it was reported that the augmentation of NK activity was not related to IFN-γ, and IL-2 alone directly acted NK activity[13]. However, most reports indicated that PBMC activated by IL-2 was belonged to NK cells and IFN-γ was related to the mechanism of this activation[4,7,8,10,11,14,16]. Thus it has not been clarified yet whether NK cells were augmented by IL-2 alone, or whether NK cells stimulated by IL-2 produced IFN-γ and NK cells were activated by their produced IFN-γ. HENNEY et al. demonstrated that NK activation by the treatment of IL-2 was augmented by IFN-γ synergistically[6], and it was reported that IFN-γ produced by NK cells stimulated IL-2 activated NK cells[20].

Moreover, SVEDERSKY et al. and ORTALDO et al. demonstrated that IL-2 partially acted the activation of NK cells and IFN-γ produced by NK cells stimulated with IL-2 partially affected the activation of NK cells[9, 15]. We have already clarified in murine model that the IL-2 receptor positive cells produced IFN-γ and the treatment of anti-IL-2 receptor Ab inhibited NK activity and the production of IFN-γ, and then IL-2 played a major role in inducing the activity NK cells by the production of IFN-γ[19].

In the present study we clarified the effect of IFN-γ to LAK activity and the mechanism of LAK activation by altering variously the conditions of the stimulation of IL-2 and IFN-γ. In PBMC of healthy subjects and cancer patients, and spleen cells of gastric cancer, the cytotoxic activity was augmented by the combination with IL-2 plus IFN-γ as compared with IL-2 or IFN-γ alone. The cytotoxic activity of PBMC of cancer patients was inhibited than that of healthy subjects by stimulations of IL-2 alone, IFN-γ or IL-2 plus IFN-γ. These results indicated that the activation of LAK cells is inhibited in cancer patients, and the combination with IFN-γ could augment the cytotoxic activity.

Washing of IL-2 from culture supernatants after 12 hours to 48 hours from the beginning of the culture did not augment the cytotoxic activity, even IFN-γ was added to the culture after washing of IL-2. Therefore, IL-2 was essential to augment the cytotoxic activity at any incubation period. On the other hand, the stimulation of IFN-γ alone from the beginning of the culture induced the same cytotoxic activity after IFN-γ was washed from the supernatants. Therefore, it was clarified that there are two different mechanisms of the activation of LAK cells by IL-2 plus IFN-γ. In existence of IL-2, the alterations of the timing and duration of addition of IFN-γ did not affect the augmentation of the cytotoxic activity that was induced without washing of IL-2. From these results, it was suggested that the lymphocytes activated by IL-2 were augmented by IFN-γ at the late incubation period and even at the early incubation period, the lymphocytes were activated by the correlation with IL-2 and IFN-γ.

We analyzed the phenotypes of the effector cells by negative selection method. The phenotypes by flow cytometry indicate the number of the effector cells only. However, by negative selection method the functional phenotypes of the effector cells are clarified in detail. From the results of the phenotypic analysis of the effector cells incubated with IL-2 and/or IFN-γ, the killer cells of healthy subjects incubated with IL-2 alone or IFN-γ alone were mainly the activated NK cells because the strong inhibitions was observed by the treatment of anti-CD2 Ab, anti-CD16 Ab and anti-Leu7 Ab. The major population of the killer cells incubated with IL-2 plus IFN-γ from PBMC of healthy subjects was CD2 positive and CD16 positive NK cells and the minor population was CD3 positive and CD8 positive cytotoxic T cells. On the other hand, the phenotype of the effector cells of cancer patients incubated with IL-2 alone, IFN-γ alone or IL-2 plus IFN-γ was only CD2 positive and CD16 positive activated NK cells, however the cytotoxic T cells was not induced in this system.

Thus, it was clarified that the phenotypes of the effector cells incubated with IL-2 alone, IFN-γ alone and IL-2 plus IFN-γ mainly the activated NK cells, however the populations of the induced effector cells differed slightly depending on the stimulation. It is suggested that IL-2 and IFN-γ may affect the various

kinds of the lymphocytes, and that the augmentation of the cytotoxic activity may be obtained.

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和文抄録

インターロイキン2とインターフェロンγのキラー活性に対する併用効果

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健常人と担癌患者末梢血および胃癌患者脾単核球のいずれにおいても，IL-2 と IFN-γ の併用によりキラー活性の増強効果を認めた．キラー活性の増強には，どの培養時期においても IL-2 の存在が必要であり，IL-2 により活性化されたリンパ球が，培養後期で IFN-γ により一層活性化を受けるのみならず，培養初期における IL-2 と IFN-γ の協調作用の機序もあることを証明した．誘導されたキラー細胞の細胞表面抗原における解析から，IL-2 と IFN-γ により活性化 NK 細胞が誘導されることを明らかにした．