

## In Vivo and in Vitro Activation of Lymphocytes by IFN- $\alpha$ and IFN- $\gamma$ in Patients with Renal Cell Carcinoma

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Treatment with interferon (IFN) for renal cell carcinoma was started in 1982 as one of the immunotherapies, but it is not so effective and no definite way of administration has been established yet. In this report for the purpose of studying the action mechanism of IFN- $\alpha$  and IFN- $\gamma$ , cytotoxic activity of peripheral blood mononuclear cells (PBMNCs) of the patients with renal cell carcinoma.

When the patients' PBMNCs were treated with IFNs at various concentrations, difference was noted in each patient referring to IFNs and their concentrations for obtaining the maximum LAK-like activity using ACHN derived from human renal cell carcinoma as target cells. Further, the combined effect of cytotoxicity obtained by IFN- $\alpha$  and IFN- $\gamma$  at their optimal concentration was additive in comparison with LAK-like activities upon therapy with IFN- $\alpha$  or IFN- $\gamma$  alone respectively. Further, when the action mechanism of IFN- $\gamma$  was studied referring to subpopulation of the normal donor' PBMNCs by cell sorting, the strong LAK-like activity was induced on the lymphocyte fraction CD3(+) CD4(-). Further in consideration of cytokine concentrations measured and the above results, it seems that IFN- $\gamma$  acts directly on the lymphocyte fraction CD3(+) CD4(-) to induce the LAK-like activity.

### INTRODUCTION

Metastasis to distant organs is the principal cause of death from renal cell carcinoma. No commonly accepted therapy is available for renal cell carcinoma with metastasis. However, it is suggested that interferon (IFN) can influence the natural course of the disease. Since 1982 when IFN was firstly used for renal cell carcinoma, the results of clinical studies investigating single agent IFN- $\alpha$  suggest that this induces objective response in about 20%<sup>1-6)</sup> of the patients with renal cell carcinoma; IFN- $\gamma$  is associated with a 20-33%<sup>6-9)</sup> response rate among these patients. In recent years as one of trials in the combination therapy with various biological response modifiers (BRM), the combination of IFN- $\alpha$  and IFN- $\gamma$  was successful, with an objective response rate of 15-50%<sup>10-14)</sup>. We studied on immunological changes and action mechanism in the patients with renal cell carcinoma under the combination therapy with IFN- $\alpha$  and IFN- $\gamma$ .

### MATERIAL AND METHOD

#### Patients

From September 1989 to September 1993, nine patients with renal cell carcinoma were treated with natural interferon- $\alpha$  (nIFN- $\alpha$ ) and recombinant interferon- $\gamma$  (rIFN- $\gamma$ ). Five patients were male and four were female. Their age ranged from 56 to 74 (mean 68.4) years. Three patients had undergone nephrectomy and six patients had progressive metastasis of renal cell carcinoma. The tumor were staged as described by Robson. One patient had stage 1, two had stage 2, and six had stage 4. None had received any cytotoxic or immunomodulatory antitumor therapy during IFN treatment.

#### Therapeutic Protocol

Patients had been treated with combination therapy of nIFN- $\alpha$  (HLBI, Sumitomo Chemical Industries, LTD., Osaka, Japan) and rIFN- $\gamma$  (OH-6000; Ohtuka Pharmaceutical, Co. Ltd., Tokushima, Japan) for 1 to several weeks. The nIFN- $\alpha$  was given intramuscular injection (dose:  $3 \times 10^6$  international units (IU)) every day and rIFN- $\gamma$  was given as continuous subcutaneous injection for 12 hours (dose:  $3 \times 10^6$  IU) every day. The nIFN- $\alpha$  was started at first and rIFN- $\gamma$  was added 7 days later.

#### Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMNCs) were isolated from heparinized blood samples of renal cell carcinoma patients by centrifugation over Ficoll-hypaque gradients. PBMNCs were washed three times and suspended in RPMI-1640 medium.

#### Assessment of natural killer (NK) and lymphokine activated killer (LAK)-like activities

Fresh PBMNCs were placed in culture wells and cultured in nIFN- $\alpha$  at concentration of  $4 \times 10^2 - 4 \times 10^4$  IU/ml or rIFN- $\gamma$  at concentration of  $5 \times 10 - 4 \times 10^3$  IU/ml. Cells were

incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air and were then washed three times in RPMI-1640 prior to the use.

Target cell lines used in this study included NK cell sensitive human myelogenous leukemia cell line K562 cells and NK resistant human renal cell carcinoma cell line ACHN cells. These cell lines were maintained in suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum.

NK and LAK-like activities were assayed by 18 hours<sup>51</sup>Cr release assay<sup>15)</sup> against K562 and ACHN. Target cells were radiolabeled with 100  $\mu$  Ci Na<sub>2</sub>CrO<sub>4</sub> at 37°C 1 hour. They were washed three times and finally resuspended 1  $\times$  10<sup>6</sup> cells/ml in complete medium.<sup>51</sup>Cr-labeled target cells (5  $\times$  10<sup>3</sup> cells/0.05ml) were dispensed into the wells of 96-well V shaped bottomed microtiter plates, after which graded numbers of effector cells were added to give effector cells to target cell ratio (E:T ratio) of 50:1, 25:1, 12.5:1, and 6.25:1. Each assay was performed in triplicate. The plates were incubated for 18 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After incubation, aliquots (100  $\mu$ l) of supernatant were removed from each well, and their radioactivity was determined in a  $\gamma$ -counter (ARC-500, Aloca, Tokyo, Japan). The data were expressed as the percentage of<sup>51</sup>Cr release, and calculated according to following formula:

$$\frac{\text{cpm. experimental release} - \text{cpm. spontaneous release}}{\text{cpm. maximal release} - \text{cpm. spontaneous release}} \times 100$$

Spontaneous release was the radioactivity release in supernatants from target cells incubated in medium only, and maximal release was determined by counting an aliquot of resuspended target cells.

One lytic unit (LU) was defined as the number of effector cells required to lysis 20% of 5  $\times$  10<sup>3</sup> target cells. The data are presented as LU/10<sup>7</sup> effector cells (Fig.1).

#### Cell sorting

Antibody in the Leu-series of Becton Dickinson Monoclonal Center Inc. (Mountain View, CA), was used. For cell sorting FACScan and FACStar (Becton Dickinson) were used. As materials for cell sorting lymphocytes obtained from 400ml of peripheral blood in the normal donors were used in various combinations with the antibody. We checked LAK-like activity of each subpopulation induced rIFN- $\gamma$  before and after sorting as follows:

- 1) PBMNCs were sorted first and 24 hours later each subpopulation was set up to culture in rIFN- $\gamma$  at 500 IU/ml for 24 hours at 37°C 5% CO<sub>2</sub> in air. Cells were washed three times and LAK-like activity was assayed against ACHN by<sup>51</sup>Cr release assay.
- 2) PBMNCs were cultured first in rIFN- $\gamma$  at 500 IU/ml for 24 hours at 37°C, 5% CO<sub>2</sub> in air and sorted. LAK-like

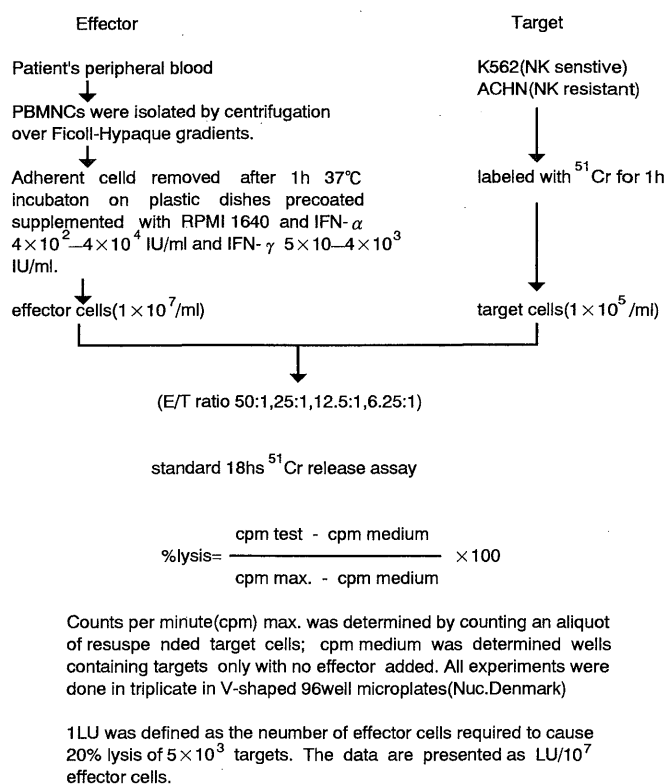


Fig.1 Experimental Method

activity was assayed against ACHN by<sup>51</sup>Cr release assay.

#### Analysis of cytokines

Cytokines (IL-1  $\alpha$ , IL-1  $\beta$ , IL-2, IL-6, IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$ ) in the serum of patients treated with nIFN- $\alpha$  and rIFN- $\gamma$  were analyzed. Serum samples were obtained patient before IFN therapy and 1, 2, 3 weeks after IFN therapy. Cytokines were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) to detect the amount of the specific antigen.

## RESULTS

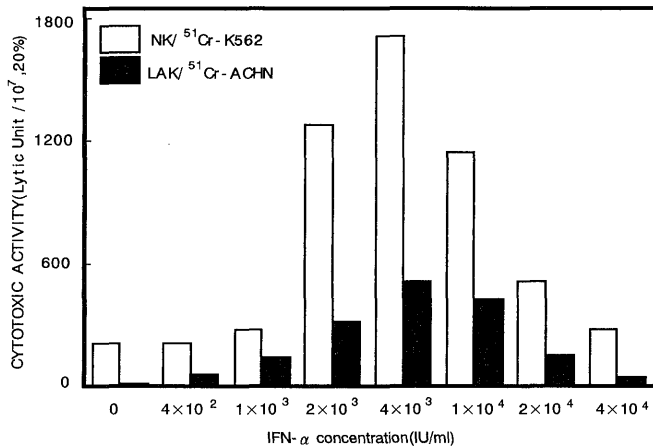
### 1) NK and LAK-like activities of PBMNCs in vitro in 9 patients with renal cell carcinoma

PBMNCs from patients with renal cell carcinoma were stimulated with nIFN- $\alpha$  or rIFN- $\gamma$  and tested in<sup>51</sup>Cr release assay against K562 and ACHN before administration of nIFN- $\alpha$  or rIFN- $\gamma$ .

*Optimal concentration of nIFN- $\alpha$  or rIFN- $\gamma$ :* As shown in Table 1, LAK-like activity increased after stimulation with nIFN- $\alpha$  or rIFN- $\gamma$  in all patients and the optimal concentration of nIFN- $\alpha$  or rIFN- $\gamma$  which induce maximal LAK-like activity differ in each patient. NK and LAK-like activities in patient were shown in Fig.2 and Fig.3.

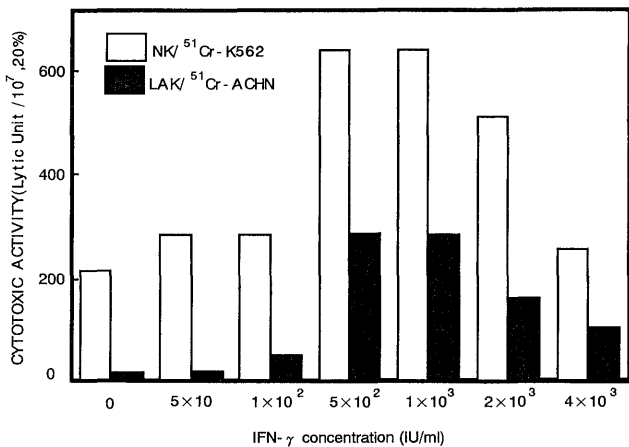
**Table 1** Concentration of IFN- $\alpha$  and IFN- $\gamma$  inducing the maximum LAK-like activity into lymphocytes in peripheral blood of the patients before starting therapy with IFN-s

patient	1	2	3	4	5	6	7	8	9
IFN- $\alpha$ (IU/ml)	$4 \times 10^3$	$2 \times 10^3$	$1 \times 10^3$	$4 \times 10^3$	$2 \times 10^3$	$4 \times 10^3$	$4 \times 10^3$	$2 \times 10^3$	$4 \times 10^3$
IFN- $\gamma$ (IU/ml)	$5 \times 10^2$	$5 \times 10^2$	$1 \times 10^3$	$5 \times 10^2$	$1 \times 10^3$	$5 \times 10^2$	$5 \times 10^2$	$5 \times 10^2$	$5 \times 10^2$



**Fig.2** NK and LAK-like activities at various IFN- $\alpha$  concentration in the induction assay for the patient with renal cell carcinoma (patient 1)

The optimal concentration was  $4 \times 10^3$  IU/ml.



**Fig.3** NK and LAK-like activities at various IFN- $\gamma$  concentration in the induction assay for the patient with renal cell carcinoma (patient 1)

The optimal concentration was  $5 \times 10^2$  IU/ml.

*LAK-like activity induced by optimal concentration of nIFN- $\alpha$  or rIFN- $\gamma$  in patients:* LAK-like activity induced by optimal concentration of nIFN- $\alpha$  or rIFN- $\gamma$  differ in patients (Table 2). The combination of nIFN- $\alpha$  and rIFN- $\gamma$  induced LAK-like activity was not synergistic but additive.

*The optimal kind of IFN:* LAK-like activity induced by

**Table 2** LAK-like activity of lymphocytes in peripheral blood of the patients with renal cell carcinoma after induction assay for 48 hours at the optimal IFN concentration

patient	1	2	3	4	5	6	7	8	9
IFN- $\alpha$ (20%LU)	512	426	512	853	426	1137	320	320	320
IFN- $\gamma$ (20%LU)	284	284	568	256	142	320	142	521	284
IFN- $\alpha$ & $\gamma$ (20%LU)	1853	646	1024	1024	853	1706	568	1137	426

nIFN- $\alpha$  was stronger than that by rIFN- $\gamma$  in patient 1, 2, 4, 5, 6, 7 and 9. LAK-like activity induced by rIFN- $\gamma$  was stronger than that by nIFN- $\alpha$  in patient 3 and 8 (Table 2). This fact showed that the optimal kind of IFN differ in patients.

2) *NK and LAK-like activities in vivo of patient treated with nIFN- $\alpha$  and rIFN- $\gamma$*

NK and LAK-like activities in vivo developed administration of nIFN- $\alpha$ . The activity was enhanced after addition of rIFN- $\gamma$  and returned after stopping administration of rIFN- $\gamma$  (Fig.4).

3) *Production of cytokines in the serum of patient treated with nIFN- $\alpha$  and rIFN- $\gamma$*

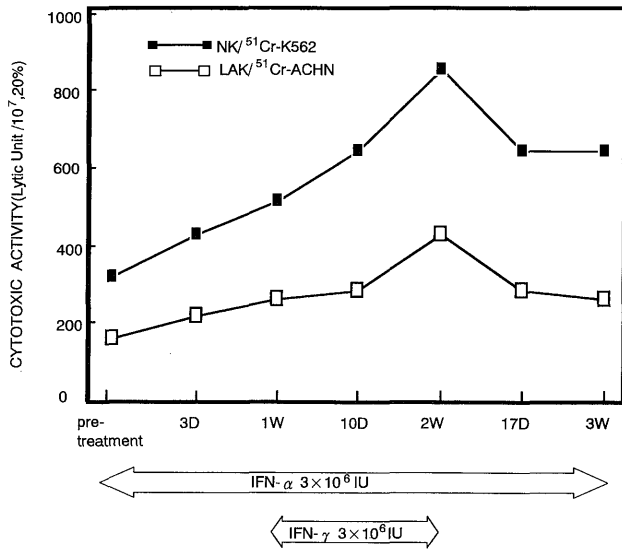
We could not detect significant elevation of IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-6, and TNF- $\alpha$  except administered IFN- $\alpha$  and IFN- $\gamma$ .

4) *Subpopulation of lymphocytes obtained by cell sorting and their LAK-like activity*

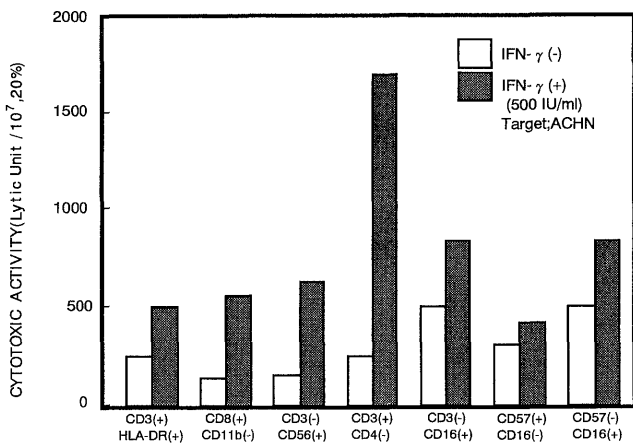
We measured the LAK-like activity of each subpopulation of PBMNCs induced by rIFN- $\gamma$ . The strong LAK-like activity was seen in the fraction CD3(+)/CD4(-) obtained by cell sorting after LAK induction assay (Fig.5). The strong LAK-like activity was also in the fraction CD3(+)/CD4(-) obtained by LAK induction assay after cell sorting (Fig.6).

**Table 3** Change of cytokine concentration with the passage of time under the combination therapy with IFN- $\alpha$  and IFN- $\gamma$  in the patient with renal cell carcinoma (patient 9)

	IL-1 $\alpha$ (pg/ml)	IL-1 $\beta$ (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)	IFN- $\alpha$ (pg/ml)	IFN- $\gamma$ (pg/ml)	TNF- $\alpha$ (pg/ml)
pretreatment	10>	20>	121	20>	20>	20>	20>
1 week	10>	20>	20>	20>	68	20>	20>
2 week	10>	20>	20>	20>	70	20>	20>
3 week	10>	20>	20>	20>	57	523	20>



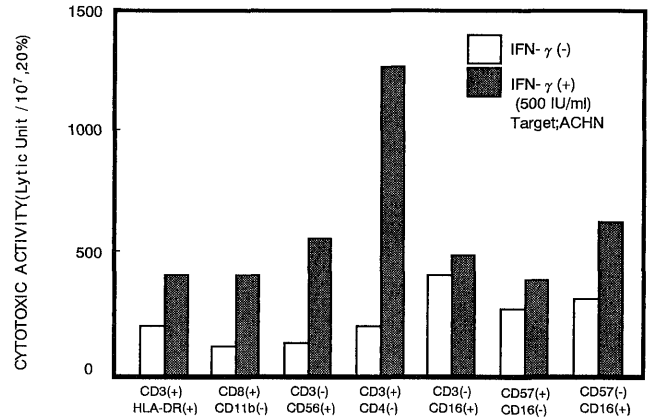
**Fig.4** Change of NK and LAK-like activities of the patient's PBMNCs under the combination therapy with IFN- $\alpha$  and IFN- $\gamma$  with passage of time (patient 9)



**Fig.5** LAK-like activity of subpopulation cultured for 24 hours in medium with and without IFN- $\gamma$  500 IU/ml after cell sorting

**DISCUSSION**

Renal cell carcinoma is comparatively rare in urological tumors, but the prognosis is not so good. The 5-year survival rate after nephrectomy is 55-65%. Death of cancer is noted frequently during the observation even in the



**Fig.6** LAK-like activity of cell sorted subpopulation after induction assay

lower stage groups expected to have the favorable prognosis after surgical treatment. Nevertheless, no established treatment is available. The immunotherapy is said to be comparatively effective but the response rate is about 20%. In recent several combination therapies of BRM have been tried. We have applied the nIFN- $\alpha$  and rIFN- $\gamma$  to patients with metastasis at first and recurrence.

PBMNCs are incubated with interferon in vitro and the killer cells which have broad spectrum are induced. Our results showed that there is an optimal concentration for individual patient to induce the maximal LAK-like activity in renal cell carcinoma-bearing patients. Further rIFN- $\gamma$  developed the higher cytotoxicity in comparison with nIFN- $\alpha$  in 2 patients, while nIFN- $\alpha$  induced the higher cytotoxicity in comparison with rIFN- $\gamma$  in other patients. This result suggests that there is an optimal kind of BRM suitable to each patient. Weigent et al<sup>16)</sup> reported that the cytotoxicity in combination use of IFN- $\alpha$  and IFN- $\gamma$  was synergistic in vitro. However, it seems to be additive in our study.

We could not detect significant elevation of IL-1  $\alpha$ , IL-1  $\beta$ , IL-2, IL-6, and TNF- $\alpha$  levels in the serum of patients undergoing nIFN- $\alpha$  and rIFN- $\gamma$  therapy. Kubota<sup>17)</sup> revealed that PBMNCs from normal healthy donors activated with nIFN- $\alpha$  in vitro showed strong LAK-like activity, while no increase of cytokines other than nIFN- $\alpha$  is noted in the supernatant of culture. Results of Kubota's experiment and this study suggest that cytotoxic

activity induced by IFN does not depend on other cytokines but IFN acts directly on the lymphocytes. Further, the strongest cytotoxicity induced by rIFN- $\gamma$  against ACHN was seen in the fraction CD3(+) CD4(-) either by the induction assay after cell sorting or by cell sorting after the induction assay. IFN- $\gamma$  seems to work directly on this fraction to induce LAK-like activity. Kubota<sup>17)</sup> demonstrated that IFN- $\alpha$  acted on the fraction CD3(-) CD16(+) to induce LAK-like activity. The subpopulation of PBMNCs stimulated by IFN- $\gamma$  is different from subpopulation stimulated by IFN- $\alpha$ . This fact showed that combination therapy with IFN- $\alpha$  and IFN- $\gamma$  may be useful for the treatment of renal cell carcinoma.

## CONCLUSION

The effect in combined use of IFN- $\alpha$  and IFN- $\gamma$  on renal cell carcinoma and action mechanisms were studied.

- (1) There seems to exist an optimal kind of BRM and an optimal concentration in each patient with renal cell carcinoma.
- (2) In vivo, the additive effect was noted on increased cytotoxic activity of PBMNCs of the patients with renal cell carcinoma upon combined use of IFN- $\alpha$  and IFN- $\gamma$ .
- (3) IFN- $\gamma$  seems to have induced the LAK-like activity to the fraction CD3(+)CD4(-) of lymphocytes in its action mechanism.

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