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Interleukin 2 induced cytotoxicity on renal cell carcinoma
2. Synergistic effects of interleukin 2 and interferon gamma

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Abstract

In the previous paper, it was shown that interleukin 2 (IL-2) enhanced NK cell activity against renal carcinoma cell lines and fresh solid tumor cells of renal cell carcinoma (RCC) which are not susceptible to NK cells, and that IL-2 induced a higher cytotoxicity than interferon γ (IFN-γ).

The present study was carried out to examine the mechanism of the enhanced cytotoxicity of IL-2 and IFN-γ in vitro, and to investigate the effects of NK cell activity when peripheral blood lymphocytes (PBL) are simultaneously treated with these lymphokines.

To test the proliferative response of PBL, ³H-TdR incorporation was determined. Effector lymphocytes, that proliferated in the presence of IL-2 but not in IFN-γ, required DNA synthesis for the induction. These lymphocytes also significantly increased phenotype in Leu 11+ cells (p<0.001).

IFN-γ at a concentration of 80 IU/ml, when added to PBL incubated with IL-2 (100 IU/ml), increases the percentage of IL-2 receptors (IL-2R) and induced a greater enhancement in NK cells activity than that incubated with IL-2 alone.

This study concluded that the combination IL-2 and IFN-γ treatment, when given to patients with RCC, might produce synergistic effects of cytotoxicity through the induction of IL-2R.

Key words: interleukin 2, interferon gamma, interleukin 2 receptor, renal cell carcinoma
Introduction

Grimm and colleagues have demonstrated the existence of a cytotoxic phenomenon distinct from natural killer (NK) cells and the conventional cytotoxic T lymphocytes (CTL). The effector cells of such an immune system have been termed lymphokine-activated killer (LAK) cells1-3 which have proven to be cytotoxic against NK-resistant solid tumor cells. LAK cells can be induced from peripheral blood lymphocytes (PBL) which are cultivated with a mitogen free interleukin 2 (IL-2) preparation and they possess different T cell antigens than those on the surfaces of NK cells. Recent investigators4,5 have shown that IL-2 receptors (IL-2R) are recognized by the anti-Tac monoclonal antibody, and that Tac antigens are not expressed on the cell surfaces of resting T lymphocytes. Thus, it will first be necessary to induce IL-2R on PBL if IL-2 is to be systemically administered to patients.

The aim of this study is to characterise killer lymphocytes induced by IL-2 and IFN-γ and to determine if IFN-γ can enhance IL-2R on PBL incubated with IL-2 in vitro. This study also aims to further investigate the possibility that a combination IL-2 and IFN-γ treatment could produce the same cytotoxic effect in patients with RCC as observed in vitro despite the small doses.

Materials and methods

The methods below were performed according to those described in the previous paper. Briefly, RPMI 1640 was used as the medium supplemented with 300 μg/l glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS). IL-2 and IFN-γ, produced by recombinant DNA technology (supplied by Shionogi Pharmaceutical Co. Ltd.) were used to generate killer effector lymphocytes. PBL from the buffy coat, obtained from healthy donors were separated by floatation on a Ficoll-Conray density gradient.6 PBL, after removing adherent cells and incubated with lymphokines, were utilized as effector cells (EC). Human renal carcinoma cell lines Caki 1,7 KU-28-9 and human leukemia cell line K56210 were employed as target cells (TC).

Analysis of T cell surface antigens: Cell surface antigens revealed by monoclonal antibodies (MoAb) were enumerated by a direct immunofluorescence assay. The fluorescein isothiocyanate (FITC) conjugated samples of MoAb against OKT 3, 4 and 8 (Ortho Pharmaceutical Co.) and Leu 7, 11 and IL-2 receptors (IL-2R) (Becton Dickinson Immunocytometry Systems Co.) were used to stain PBL. The expression of lymphocyte subpopulations and IL-2R on cell surfaces were determined using FITC-labeled MoAb and a flow cytometer (Spectrum 3, Ortho Co.). 10^6–10^7 cells/ml of PBL, preincubated with lymphokines, were incubated with 10 μl of 40 μg/ml of MoAb for 45 min at 4°C. After washing twice, the cells were brought up in 1.0 ml phosphate-buffered saline for flow cytometry.
3H-TdR incorporation: 0.5 μCi of 3H-TdR (New England Nuclear) was put into each flat bottomed microtiter plate well, which contained 2.0×10⁴ cells/well, 7 hr before harvesting the cells. The cells were collected on glass fiber filters using an automatic harvester, and 3H-TdR incorporation was determined in triplicate by a liquid scintillation counter.

NK assay: NK cell activity was determined by 51Cr-release assay as previously reported. For the assay, TC were labeled with 100 μCi of 51Cr solution (Dai-ichi Radioisotope Laboratories, Ltd.) for 1 hr, washed twice and plated out at 10⁴ cells/well in 96-well, round bottomed microplates. After incubation at 37°C for 4 hr at 50:1 of the EC:TC ratio, 100 μl of supernatant were collected from each well and the radioactivity was counted in a γ counter. Cytotoxicity was calculated with the formula below:

\[
\% \text{ Cytotoxicity} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100
\]

Spontaneous release was considered to be cpm released from TC in the medium alone, and the maximum release from TC treated with 100 μl of 5% triton X. The values are presented as the mean of triplicate samples.

Statistical analysis: The values present the mean ± SD of different healthy donors. A p value of less than 0.05 was accepted as significant using the Student t-test.

Results

Subpopulation of IL-2 induced killer cells: The influence of IL-2 on PBL subpopulations were detected using MoAb against OKT 3, OKT 4, OKT 8, Leu 7 and Leu 11 detected by flow cytometry. As shown in Table 1, there were distinctly increased percentage of Leu 11+ cells expressed on the surfaces of PBL incubated with 100 IU/ml of IL-2 for 72 hr from 8.3 ± 4.9% to 20.0 ± 5.2% (p<0.001). It was also demonstrated that IFN-γ didn't significantly influence the phenotype in the Leu 11+ cell (Fig. 1).

Proliferation of IL-2 induced killer cells: To study the mechanism of NK-activation by IL-2, the effects of IL-2 and IFN-γ on the proliferative response of PBL were measured by 3H-TdR incorporation (Fig. 2). IL-2, but not IFN-γ, significantly increased 3H-TdR incorporation into lymphocytes (p<0.01), indicating that IL-2 induced killer cells required DNA synthesis for their activation.

Combination IL-2 and IFN-γ treatment on cytotoxicity: Enhanced cytotoxicities by combination treatment are shown in Fig. 3, 4 and 5. PBL were incubated with 100 IU/ml of IL-2 for 24 hr and/or 80 IU/ml of IFN-γ for 24 hr. At a low concentration (80 IU/ml) of IFN-γ, the satisfactory ability to lyse renal carcinoma cell lines wasn’t observed as shown in the previous experiment. It was demonstrated that simultaneous treatment with IL-2 and IFN-γ led to the highest amount of cytotoxicity.
Fig. 1 Effects of IL-2 and IFN-γ on Leu 11 expression. After peripheral blood lymphocytes from fourteen different healthy donors were incubated with 100 IU/ml of IL-2 for 72 hr, or with 400 IU/ml of IFN-γ for 48 hr, the percentage of Leu 11+ cells was determined through flow cytometry.

(46.7 ± 8.5% against Caki 1 and 50.0 ± 4.8% against KU-2) that could not be enhanced by IL-2 or IFN-γ alone. The combined effect is higher than the sum of the single effect of IL-2 and IFN-γ (34.7 ± 8.8% against Caki 1 and 37.3 ± 8.2% against KU-2, p<0.05).

Induction of IL-2R by IFN-γ: The effects of IFN-γ and/or IL-2 on IL-2R expression were shown using the combination method of anti IL-2R MoAb and flow cytometry (Fig. 6). There were no IL-2R on resting PBL or IFN-γ incubated PBL, and IFN-γ at a concentration of 80 IU/ml, when added to PBL incubated with IL-2 (100 IU/ml) for 48 hr, increased the percentage of IL-2R from 57% to 93%.
Fig. 2  Effects of IL-2 and IFN-γ on proliferative response of their induced killer lymphocytes. After lymphocytes from nine different healthy donors were incubated with 100 IU/ml of IL-2 for 72 hr or with 400 IU/ml of IFN-γ for 48 hr, ³H-TdR incorporation was determined by a liquid scintillation counter.

Table 1

*Effects of IL-2 on lymphocyte subpopulations*¹)

<table>
<thead>
<tr>
<th>MoAb⁵)</th>
<th>Subpopulation (%)</th>
<th>Control¹)</th>
<th>IL-2</th>
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<tbody>
<tr>
<td>OKT 3</td>
<td>66.00±10.38</td>
<td>66.57±8.75 (N.S.)</td>
<td></td>
</tr>
<tr>
<td>OKT 4</td>
<td>38.00±8.16</td>
<td>36.71±6.73 (N.S.)</td>
<td></td>
</tr>
<tr>
<td>OKT 8</td>
<td>29.43±7.40</td>
<td>30.71±8.40 (N.S.)</td>
<td></td>
</tr>
<tr>
<td>Leu 7</td>
<td>29.43±8.42</td>
<td>31.71±6.92 (N.S.)</td>
<td></td>
</tr>
<tr>
<td>Leu 11</td>
<td>8.33±4.85</td>
<td>20.00±5.18 (P&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

*¹) Lymphocytes were incubated with 100 IU/ml of IL-2 for 72 hr. Values represent the means ± SD of fourteen different donors.

*⁵) Monoclonal antibodies.

*²) Lymphocytes incubated in complete medium for 72 hr.*
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Fig. 3 Synergistic augmentation of cytotoxicities against Caki 1 by combination IL-2 and IFN-γ treatment. Lymphocytes from six different healthy donors were incubated with 100 IU/ml of IL-2 or 80 IU/ml of IFN-γ alone, or with mixture of these lymphokines for 24 hr. Control indicated that lymphocytes were incubated with complete medium for 24 hr. Effector/target cells ratio was 50:1.

Fig. 4 Synergistic augmentation of cytotoxicities against KU-2 by combination IL-2 and IFN-γ treatment. Lymphocytes from six different healthy donors were incubated with 100 IU/ml of IL-2 or 80 IU/ml of IFN-γ alone, or with mixture of these lymphokines for 24 hr. Control in the absence of any reagents but medium for 24 hr. Effector/target cells ratio was 50:1.
Discussion

IL-2 has been required for the function and proliferation of T lymphocytes, and for the induction of NK cells and cytotoxic effector cells containing LAK cells in vitro and in vivo. LAK phenomenon, as previously described, can readily be generated by incubating fresh PBL with IL-2.

The previous paper indicated that IL-2 enhanced cytotoxicity against renal carcinoma cell lines, the effects of which were dose and time dependent and that LAK activity exceeded the levels of NK cell activity cultivated in PBL with IFN-γ.

For the treatment of advanced RCC which is resistant to IFN-γ activated NK cells, systemic IL-2 therapy or adoptive immunotherapy utilizing LAK cells may produce a great deal of effects.

In the present study, the characteristics of effector killer lymphocytes induced by IL-2 (LAK cells) were compared to those induced by IFN-γ, and the enhanced cytotoxicity levels were measured when PBL were simultaneously cultured in IL-2 and IFN-γ.
The present data showed that simultaneous IL-2 and IFN-γ treatment resulted in synergistic effects on the enhancement of NK cell activity against renal carcinoma cell lines although these lymphokines could generate killer cells with different qualities in vitro.

Earlier studies have shown that IL-2 induced cytotoxicity is enhanced by IFN-γ produced by IL-218 and disclosed to be partially neutralized by anti IFN-γ.18,19 The IL-2 induced enhancement, however, did not occur solely due to endogenously produced IFN-γ because treatment with excessive IFN-γ didn’t achieve the same levels of NK cell activity. Other investigators have indicated that IFN-γ activates PBL to make them responsive to IL-2, possibly via increased expression of IL-2R.19,20

A further investigation was carried out to see if PBL exposed to IL-2 and/or IFN-γ
could express IL-2R using the combination method of a direct immunofluorescence technique and flow cytometry using anti IL-2R MoAb. It was also shown that the percentage of IL-2R was increased when PBL were simultaneously incubated with IL-2 and IFN-γ, compared to the incubation with only one.

Itoh et al.\textsuperscript{20} have shown that IFN-γ cultures of PBL without any other reagents could successfully increase Tac antigen expression, which was originally prepared by Uchiyama et al.\textsuperscript{4,5} and recognized the human IL-2R on cell surfaces. However, it was observed that there was no IL-2R expression detectable on PBL, either before or after incubation with 80 IU/ml of IFN-γ for 24 hr and even when a high dose IFN-γ (400-1,600 IU/ml) was used for the IL-2R induction for 24 hr or 48 hr in vitro (data not shown). The obtained results led to the conclusion that IFN-γ had a twin effect on NK cells. Firstly, it directly enhanced its cytotoxicity, and secondly it enhanced the affinity of IL-2 to NK cells by inducing IL-2R expression on cell surfaces. It was also demonstrated PBL incubated with IL-2 alone expressed its own receptors (IL-2R) \textit{in vitro}. Svedersky et al.\textsuperscript{21} have shown NK cell activity is increased after only 5 min exposure to IL-2, and it was of great interest to determine whether IL-2R could appear on PBL when they were exposed to IL-2 for quite a short time.

Phenotype on cell surfaces of PBL were measured by flow cytometry. When PBL were incubated with 100 IU/ml of IL-2 for 72 hr, IL-2 increased the percentage of Leu 11^+ cells, which have been proven to react with the Fc-receptor of NK cells and granulocytes, and is specific to active NK cells. Although Leu 7 is also specific to NK cells, recent studies have revealed that Leu 7^- Leu 11^+ cells, separated by a cell sorter, possess the highest cytotoxicity\textsuperscript{22} and are the most responsive to IL-2 exposure among subpopulations.\textsuperscript{23,24} Leu 11^+ cells with NK cell activity might be percursor of IL-2 induced killer cells. On the other hand, the results showed that those effecter lymphocytes were induced accompanied with an increase of Leu 11^+ cells, indicating that Leu 11 bearing NK cells were enhanced in terms of their cytotoxicity by IL-2, at least, in killing phases.

PBL incubated with IL-2 seemed to require DNA synthesis for the induction. The characteristics of LAK cells were similar not to NK cells activated by IFN-γ but to conventional CTL. It was conceivable that these lymphokines interacted and collaborated with each other to produce higher cytotoxicity \textit{in vitro} and possibly \textit{in vivo} although the mechanism of their enhancement in cytotoxicity were different on the proliferation of killer lymphocytes and the expression of cell surface antigens. The other paper described the possibility that systemic administration of IL-2 will be helpful in the treatment of advanced RCC by inducing LAK activity. However, there are lots of problems to be resolved, such as toxic side effects because of the excessive doses of IL-2 required to maintain levels over 4 IU/ml in the serum. It's also necessary to induce IL-2R, which are not expressed on resting lymphocytes, when this lymphokine is practi-
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cally administered to patients. To solve these problems, this study was accomplished to suggest that the combined therapy of IL-2 and IFN-γ had the advantages. This therapy could induce more IL-2R than IL-2 therapy alone and would need neither high doses of the lymphokines nor long exposure time. Thus, it is expected that the combination IL-2 and IFN-γ therapy will be used in clinical cases based on these experimental results and will hopefully produce the same cytotoxic effects as seen in vitro.

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