SYNERGISTIC ANTICELLULAR AND ANTIVIRAL ACTIVITIES OF HUMAN RECOMBINANT INTERFERON- γ AND - β

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The synergism of anticellular and antiviral activities of recombinant human interferon- γ (ReIFN- γ) and recombinant human interferon- β (ReIFN- β) was examined *in vitro* using human melanoma SK-MEL-28 cells. Some differences were detected in the kinetics of anticellular activity between both IFNs, namely the inhibitory effect of ReIFN- β occurred earlier than that of ReIFN- γ . Significant synergism was detected in the anticellular activity of both IFNs when growth curves and isobolograms were examined. A difference between ReIFN- γ and ReIFN- β was also detected in antiviral activity. The antiviral activity of ReIFN- γ against vesicular stomatitis virus (VSV) was significantly weaker than that of ReIFN- β , even though both IFNs exhibited almost equivalent antiviral activities against Sindbis virus. However, ReIFN- γ and ReIFN- β exhibited synergistic antiviral activities against both VSV and Sindbis virus. The analysis of cell cycle distribution by flow cytometry revealed that there were some differences in the distribution pattern between cells treated with ReIFN- γ alone, ReIFN- β alone, or ReIFN- γ and ReIFN- β in combination. ReIFN- β induced a prolongation or accumulation of S phase, whereas the effect of ReIFN- γ was cycle-nonspecific. The combination of ReIFN- γ and ReIFN- β induced a decrease of G₁ phase and an increase of G₂M phase.

These results suggest that ReIFN- γ and ReIFN- β used in combination were more effective in inhibiting the growth of human tumor cells and the proliferation of viruses than IFN used individually.

Keywords — interferon- β ; interferon- γ ; recombinant; anticellular; antiviral; synergism; cell cycle

INTRODUCTION

Among the three different types of natural interferons (IFNs), interferon- α (IFN- α), interferon- β (IFN- β) and interferon- γ (IFN- γ), IFN- γ was reported to be most efficient in inhibiting growth of cultured cells and to be cytotoxic in some cases.¹⁾ The antiviral activity of IFN- γ was also reported to show different characteristics from that of IFN- α or IFN- β in terms of kinetics²⁾ and spectrum.^{1a)}

We previously reported that human IFN- β was efficient in inhibiting the growth of human melanoma cell lines *in vitro* and *in vivo* transplanted into nude mice.^{3a),b)} These results correlated well with the efficiency of IFN- β against human melanomas in a clinical trial.^{3c)} Since the anticellular activity of recombinant human interferon- β (ReIFN- β) against human melanoma cell lines was almost equivalent to that of IFN- β *in vitro*^{3a)} and *in vivo*,^{3b)} and the *in vitro* anticellular activity of recombinant human interferon- γ (ReIFN- γ) against human melano-

ma cell lines was almost equivalent to that of ReIFN- β ,⁴⁾ it seemed to be worthwhile to study the synergism of *in vitro* anticellular activity of both IFNs. It was previously reported that IFN- γ exhibited synergistic anticellular and antiviral activities with IFN- α or IFN- β .⁵⁾

The present study was carried out in order to compare the characteristics of anticellular and antiviral activities of ReIFN- γ with those of ReIFN- β and to investigate the synergism of anticellular and antiviral activities of ReIFN- γ and ReIFN- β .

MATERIALS AND METHODS

Cells and Culture — Human melanoma SK-MEL-28 cells⁶⁾ and human uterine cervix carcinoma HeLa S₃ cells⁴⁾ were grown and maintained in Eagle's minimal essential medium (MEM, Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.).

Interferons — ReIFN- γ , a recombinant human interferon- γ produced by *Escherichia coli* in our laboratories,^{7d)} had a purity of over 99% and a specific activity of over 5×10^6 Japanese reference units (JRU)/mg protein. The titer of ReIFN- γ was expressed as JRU/ml of Japanese ReIFN- γ reference (J-Rr40101, National Institute of Health, Tokyo, Japan), which had been established based on the international units (IU) of WHO IFN- γ reference (Gg23-901-530). ReIFN- β , a recombinant human interferon- β produced by *E. coli* in our laboratories, had a purity of over 99.5% and a specific activity of over 5×10^7 IU/mg protein.^{7a-c)} The titer of ReIFN- β was expressed as IU/ml of reference IFN- β (J-Ref-02, National Institute of Health, Tokyo, Japan).

Evaluation of Anticellular Activity — SK-MEL-28 cells (10^4 /ml) were cultured in 24-well multidishes (Nunc, Roskilde, Denmark), and IFNs were added on day 1. On the indicated days, cells were washed with Dulbecco's phosphate-buffered saline (Ca^{2+} —, Mg^{2+} —free, PBS (—)), incubated with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) (Grand Island Biological Co.), suspended by pipetting and counted with a Toa micro-cell counter (Toa Medical Electronics Co., Hyogo, Japan).^{3a,4)} The IC_{50} (interferon concentration required for 50% growth inhibition) values were calculated from the cell numbers of triplicate cultures at each concentration by the Reed-Münch method.

Cell Cycle Analysis — SK-MEL-28 cells (1.5×10^6 /30 ml) were cultured in plastic flasks (Nunc), and IFNs were added on day 1. At the time indicated, cells were harvested after treatment with 0.02% EDTA, fixed by 70% ethanol solution, hydrolyzed with 170 $\mu\text{g}/\text{ml}$ of ribonuclease A (type 1-A, Sigma, St. Louis, M.O.) at 37 °C for 30 min and stained with propidium iodide (Sigma). The nuclei were analyzed with a Coulter EPICS V cell sorter (Coulter Electronics, Inc., Hialeah, Fla.). Analysis of cell cycle distribution was carried out using the method of Bagwell's algorithm.⁸⁾

Antiviral Activity — The antiviral activities of IFNs were determined by the yield reduction method⁹⁾ using vesicular stomatitis virus (VSV, New Jersey strain) and Sindbis virus. SK-MEL-28 cells (1.5×10^5 /well) seeded in 24-well multidishes on day 0 were treated with IFNs and

challenged with 100 TCID_{50} (50% tissue culture infectious dose) of VSV or Sindbis virus at the time indicated. After virus adsorption of 37 ° for 2 h, the cells were washed twice with PBS (—) and further incubated in MEM containing 2% fetal bovine serum in the case of VSV or MEM without fetal bovine serum in the case of Sindbis virus. After 24 h, the cells were frozen and thawed, and virus titers of culture supernatant fluids were measured by the cytopathic effect of each virus against HeLa S₃ cells, which were pre-cultured at 1.5×10^4 cells/well in 96-well flat bottom microplates (Nunc) for 2 d. Virus titer was expressed as the magnification of virus dilution required for 50% cytopathic effect against HeLa S₃ cells.

Analysis of Synergism — The synergism of ReIFN- γ and ReIFN- β was analyzed by calculating the expected values and analysis of isobolograms, respectively.¹⁰⁾ The expected value was calculated by multiplying the effect of ReIFN- γ alone with that of ReIFN- β alone. The isobolograms were generated from IC_{50} (%) of both IFNs calculated against IC_{50} value (JRU or IU/ml) of each IFN added alone. If the observed data were under the expected values or the isobolograms were closer to the origin of the coordinate axes than to the diagonal line which indicated the additive effect, the effect of both IFNs was considered to be synergistic.

RESULTS

Growth Curve of SK-MEL-28 Cells Treated with ReIFN- γ or ReIFN- β

In order to compare the cell growth-inhibitory activity of ReIFN- γ and ReIFN- β , SK-MEL-28 cells seeded on day 0 were treated with IFNs on day 1, and the cell numbers were counted daily (Fig. 1). Both IFNs inhibited the growth of SK-MEL-28 cells at concentrations of over 10^2 JRU or IU/ml. However, there were some differences in the kinetics of anticellular activity between both IFNs. Namely, the inhibitory effect of ReIFN- β occurred earlier than that of ReIFN- γ .

Synergism of Anticellular activity of ReIFN- γ and ReIFN- β

In order to investigate the combined effect of ReIFN- γ and ReIFN- β , SK-MEL-28 cells were seeded on day 0, treated with both IFNs on day 1, and cells were counted on days 3, 5, 7 and 9 (Fig. 2). The observed effect of both IFNs used

FIG. 1. Growth Curves of SK-MEL-28 Cells Treated with ReIFN- γ or ReIFN- β

SK-MEL-28 cells (10^4 /ml) seeded on day 0 were treated with 10 ($\bullet$), 10^2 ($\blacktriangledown$) or 10^3 ($\blacksquare$) JRU/ml of ReIFN- γ (A) or IU/ml of ReIFN- β (B) on day 1, and cells were counted on each day. $\circ$, untreated control group.

FIG. 2. Growth Curves of SK-MEL-28 Cells Treated with ReIFN- γ and ReIFN- β in Combination

SK-MEL-28 cells (10^4 /ml) seeded on day 0 were treated with ReIFN- γ alone ($\bullet$), ReIFN- β alone ($\blacktriangledown$), or both IFNs in combination ($\blacksquare$) on day 1, and cells were counted on each day. 10^2 IU/ml of ReIFN- β were added in combination with 10 (A), 10^2 (B) or 10^3 (C) JRU/ml of ReIFN- γ , or 10^2 JRU/ml of ReIFN- γ were added in combination with 10 (D), 10^2 (E), or 10^3 (F) IU/ml of ReIFN- β . $\circ$, untreated control group. $\square$, expected cell number of combination group, which was calculated from the cell number of a group treated with ReIFN- γ alone and that treated with ReIFN- β alone.

FIG. 3. Synergism of Anticellular Activity of ReIFN- γ and ReIFN- β

SK-MEL-28 cells (10^4 /ml) were seeded on day 0, and fixed concentrations of ReIFN- γ ($\circ$) or ReIFN- β ($\bullet$) were added with increasing concentrations of ReIFN- β ($\circ$) or ReIFN- γ ($\bullet$), respectively, on day 1. On day 5, cells were counted, and IC_{50} (%) was calculated against IC_{50} value of each IFN added alone, and plotted as the isobolograms. IC_{50} values of ReIFN- γ and ReIFN- β used alone were 835 JRU/ml and 545 IU/ml, respectively.

in combination was compared with the expected values which were calculated by multiplying the effect of ReIFN- γ alone with that of ReIFN- β alone. As the observed growth of SK-MEL-28 cells treated with ReIFN- γ and ReIFN- β in combination was inhibited more significantly than the expected growth (Fig. 2 (B, C, E, F)), the anticellular activity of ReIFN- γ and ReIFN- β used in combination at concentrations of over 10^2 JRU or IU/ml was found to be synergistic. This synergism was most significant on day 5, 4 d after the addition of both IFNs.

The synergism of anticellular activity of ReIFN- γ and ReIFN- β was also confirmed by the analysis by isobolograms (Fig. 3). Since the plots of the isobolograms were much closer to the origin of the coordinate axes than to the diagonal line, which indicated the additive effect, the effect of ReIFN- γ and ReIFN- β used in combination was found to be significantly sy-

FIG. 4. Effect of ReIFN- γ and ReIFN- β on Cell Cycle Distribution of SK-MEL-28 Cells

SK-MEL-28 cells (5×10^4 /ml) seeded on day -1 were treated with 10^3 JRU/ml of ReIFN- γ ($\bullet$), 10^3 IU/ml of ReIFN- β ($\blacksquare$), or both IFNs in combination ($\square$) on day 0. $\circ$, untreated control group. G_1 , S and G_2M phases are shown in (A), (B) and (C), respectively.

nergistic.

Effect of ReIFN- γ and ReIFN- β on Cell Cycle Distribution

The effect of IFNs on cell cycle distribution of asynchronous SK-MEL-28 cells was analyzed by flow cytometry (Fig. 4). In ReIFN- β -treated cells, the percentage of cells in G_1 phase decreased 24 h after treatment and the percentage of cells in S phase increased. These changes continued for 3 or 4 d. In ReIFN- γ -treated cells, significant changes were not detected, although the percentage of cells in S phase decreased slightly

FIG. 5. Antiviral Activity of ReIFN- γ and ReIFN- β against VSV

SK-MEL-28 cells (1.5×10^5 /ml) seeded on day 0 were treated with 1 (b), 10 (c) or 10^2 (d) JRU/ml of ReIFN- γ or 1 (e), 10 (f) or 10^2 (g) IU/ml of ReIFN- β on day 1, and challenged with VSV on day 2. After 24 h, titers of VSV (■) were determined using HeLa S_3 cells as described in Materials and Methods. a, untreated control group. □, expected titer of combination group, which was calculated from the titer of a group treated with ReIFN- γ alone and that treated with ReIFN- β alone. Bars, S. D.

between 3 and 4 d after treatment. When the cells were treated with ReIFN- γ and ReIFN- β in combination, the percentage of cells in G_1 phase decreased 3 d after treatment, whereas that in G_2 M phase increased.

Synergism of Antiviral Activities of ReIFN- γ and ReIFN- β

Synergism of ReIFN- γ and ReIFN- β was examined in terms of antiviral activity against VSV (Fig. 5) or Sindbis virus (Fig. 6) by the yield reduction method using SK-MEL-28 cells. Against VSV, ReIFN- β exhibited dose-dependent antiviral activity, and at the concentration of 10^2 IU/ml the proliferation of VSV was almost completely inhibited (Fig. 5). On the other hand, the antiviral activity of ReIFN- γ against VSV was not so significant, and even at the concentration of 10^2 JRU/ml, the proliferation of VSV was detected. When 10, 10^2 and 10^3 JRU/ml of ReIFN- γ were used in combination

FIG. 6. Antiviral Activity of ReIFN- γ and ReIFN- β against Sindbis Virus

SK-MEL-28 cells (1.5×10^5 /ml) cultured for 44 h were treated with 0.1 (b), 0.3 (c), 1 (d) or 3 (e) JRU/ml of ReIFN- γ or 0.1 (f), 0.3 (g) or 1 (h) IU/ml of ReIFN- β for 4 h, and challenged with Sindbis virus. After 24 h, titers of Sindbis virus (■) were determined using HeLa S_3 cells as described in Materials and Methods. a, untreated control group. □, expected titer of combination group, which was calculated from the titer of a group treated with ReIFN- γ alone and that treated with ReIFN- β alone. Bars, S. D.

with 10, 1 and 10 IU/ml of ReIFN- β , respectively, synergistic antiviral activity was detected, since the observed titers of VSV in groups treated with ReIFN- γ and ReIFN- β in combination were lower than the expected titers. Against Sindbis virus, both ReIFN- γ and ReIFN- β exhibited significant antiviral activity, and at the concentration of 3 JRU/ml of ReIFN- γ or 1 IU/ml of ReIFN- β the proliferation of Sindbis virus was inhibited completely (Fig. 6). When 0.1–1 JRU/ml of ReIFN- γ were used in combination with 0.1–0.3 IU/ml of ReIFN- β , respectively, significant synergistic antiviral activity was detected. These results indicate that ReIFN- γ and ReIFN- β exhibited synergistic antiviral activity against both VSV and Sindbis virus.

DISCUSSION

Natural human interferon- β (IFN- β) was reported to be efficient at inhibiting the growth

of human melanoma cell lines *in vitro* and *in vivo* transplanted into nude mice.^{3a,b)} These results are suggested to correlate with the efficiency of IFN- β against human melanomas in a clinical trial.^{3c)} Since the anticellular activity of ReIFN- β against human melanoma cell lines was almost equivalent to that of IFN- β *in vitro*^{3a)} and *in vivo*,^{3b)} the deficiency of carbohydrate in ReIFN- β was suggested not to affect anticellular activity. The *in vitro* anticellular activity of ReIFN- γ was shown to be more significant than those of IFN- α or ReIFN- β , especially, against human carcinoma and sarcoma cell lines.⁴⁾ Therefore, it was worthwhile to compare the anticellular activity of ReIFN- γ and ReIFN- β against human melanoma cell lines and furthermore to examine the *in vitro* anticellular activity of both IFNs used in combination.

Both ReIFN- γ and ReIFN- β inhibited the growth of SK-MEL-28 cells at concentrations of over 10^2 JRU or IU/ml (Fig. 1). However, some differences were detected in the kinetics of anticellular activity between both IFNs, namely ReIFN- β inhibited the growth of cells more rapidly than ReIFN- γ . Significant synergism was seen in the anticellular activity of both IFNs when the growth curves (Fig. 2) and isobolograms (Fig. 3) were examined. The difference between ReIFN- γ and ReIFN- β was also detected in the antiviral activity. Namely, the antiviral activity of ReIFN- γ against VSV was not so significant as that of ReIFN- β (Fig. 5), although both IFNs exhibited almost equivalent antiviral activity against Sindbis virus (Fig. 6). However, ReIFN- γ and ReIFN- β exhibited synergistic antiviral activity against both VSV and Sindbis virus.

One of the mechanisms of synergism of anticellular activity of ReIFN- γ and ReIFN- β was the difference in the kinetics of anticellular activity of both IFNs (Fig. 1). Since ReIFN- β inhibited the growth of SK-MEL-28 cells more rapidly than ReIFN- γ , the combined use of both IFNs might be more effective to inhibit the growth of the cells. Another mechanism was the difference in the receptors of ReIFN- γ and ReIFN- β . While SK-MEL-28 cells possess common receptors for ReIFN- β and IFN- α , the receptors for ReIFN- γ are independent of those of ReIFN- β or IFN- α (in preparation for submission). This difference in receptors may correlate with the different responses induced in IFN-

treated cells. ReIFN- β significantly enhanced 2'-5' oligoadenylate synthetase activity in cultured cells, whereas ReIFN- γ barely did so.¹¹⁾ On the other hand, ReIFN- γ possessed a cellular protein-inducing activity and the synthesis of several proteins was induced or enhanced in ReIFN- γ -treated cells, whereas this activity of ReIFN- β was not so significant (in preparation for submission). Thus, the cellular responses induced by ReIFN- γ are different from those by ReIFN- β ; consequently the combined use of ReIFN- γ and ReIFN- β demonstrated synergistic anticellular and antiviral activities.

The different antiviral activities of ReIFN- γ and ReIFN- β against VSV might be explicable by the difference in 2'-5' oligoadenylate synthetase-inducing activity. 2'-5' oligoadenylate was reported to be one of significant mechanisms responsible for the antiviral resistance of IFN-treated cells against VSV.¹²⁾ Since 2'-5' oligoadenylate synthetase-inducing activity of ReIFN- γ was not so active as that of ReIFN- β ,¹¹⁾ the antiviral activity of ReIFN- γ against VSV is not so potent as that of ReIFN- β (Fig. 5). Although both IFNs exhibited significant antiviral activity against Sindbis virus, we do not yet understand the mechanisms responsible for this antiviral activity. Further study is required.

Many experiments have been reported concerning the effect of IFN- α or IFN- β on cell cycle distribution.¹³⁾ However the results differed according to experimental conditions. While the effects of IFN- α or IFN- β were reported to be cycle-nonspecific in some cases,^{13a,b)} the prolongation or accumulation of the S phase was reported in other cases.^{13c,d)} Our results are in accordance with the later, since the treatment of SK-MEL-28 cells with ReIFN- β increased the percentage of cells in the S phase (Fig. 4). The effects of IFN- γ on cell cycle distribution have not been examined as extensively as those of IFN- α or IFN- β .¹⁴⁾ The effects of IFN- γ was reported to be cycle-nonspecific^{14b)} or to prolong the G₁ phase.^{14a)} In SK-MEL-28 cells, the effect of ReIFN- γ was not significant, suggesting that its effect was cycle-nonspecific. As for the effects of IFNs used in combination on the cell cycle distribution, few studies have been reported. Our results indicated that the combination of ReIFN- γ and ReIFN- β induced the synergistic anticellular activity significantly (Figs. 2 and 3), the decrease of G₁ phase 3 d after treatment and

the increase of G₂M phase. These results suggest that synergism of anticellular activity of ReIFN- γ and ReIFN- β might be induced by the inhibition of mitosis of the cells. Further investigations are planned to study the inhibition of mitosis by IFNs.

In conclusion, ReIFN- γ and ReIFN- β exhibited significant synergism to elicit anticellular and antiviral activities, suggesting that the combined use of ReIFN- γ and ReIFN- β is a reasonable therapy for both cancer and viral diseases.

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