In Vivo Antitumor Activity of Multiple Injections of Recombinant Interleukin 2, Alone and in Combination with Three Different Types of Recombinant Interferon, on Various Syngeneic Murine Tumors

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ABSTRACT

We have used several transplantable experimental murine tumors to evaluate the potentiation of antitumor activity by a combination of human recombinant interleukin 2 (rHIL2) and recombinant interferons (rIFNs). The combination of rHIL2 and either human hybrid recombinant α-interferon A/D (rIFN-α A/D) or mouse recombinant β-interferon (rIFN-β) induced the s.c. adenocarcinoma 755, which had been established for 8 days, to regress, although rHIL2 or the rIFNs alone hardly inhibited the tumor’s growth. Eight injections of the rHIL2-rIFN-α A/D combination cured 38% of the tumor-bearing mice. The rHIL2-rIFN-β combination achieved a complete cure only when given in more than 13 injections. The administration of rHIL2 and mouse recombinant γ-interferon (rIFN-γ) markedly inhibited tumor growth of the s.c. established adenocarcinoma 755, but did not cure any of the mice. Other tumors, B16-F10 melanoma, and colon tumors 38 and 26 responded almost as well to a rHIL2-rIFN-α A/D or -β combination, but not to a rHIL2-rIFN-γ combination. The growth of Lewis lung carcinoma was inhibited to a lesser extent by all combinations, for which there were no long-term survivors.

The combination therapy of rHIL2 and rIFN-β produced a marked regression of the tumor in beige mice which have low natural killer activity, suggesting the activated natural killer cells not to be responsible for the therapeutic effect. And T-cell immunity may be important in the regression of s.c. established tumors, because of the lesser potentiation of antitumor activity in athymic mice.

These results demonstrate that combination therapies of rHIL2 and rIFN-α A/D or -β can function synergistically in the various s.c. established murine tumor systems and give further evidence in support of their clinical potential.

INTRODUCTION

IL2, a protein released by activated helper T-lymphocytes (1, 2), is essential for the expansion of antigen-triggered T-lymphocytes and cytotoxic T-cells, which are frequently depressed in malignancy. IL2 exerts its growth-promoting properties after interacting with specific membrane binding site (IL2 receptors) expressed on activated, but not on resting, T-cells (3-5). The cytotoxic T-cells, expanded in IL2, are shown to retain in vivo antitumor activity (6-8). In addition to its effect on T-cells, IL2 also promotes the growth of NK cells (9) and enhances murine NK cytotoxic activity in vivo (10). IFNs have been shown to augment NK activity in vitro and in vivo (11-13) and found to have therapeutic activity against certain types of leukemias and lymphomas, as well as against renal cell carcinoma (14-17); their efficacy against most other human solid tumors has, however, been limited (18, 19). Adequate evaluation of IFN’s therapeutic efficacy in animal models was hampered until recently by a lack of sufficient purified murine materials. The advent of recombinant DNA technology has made it possible to produce several species of recombinant IFN in large quantities. When combined with IL2, IFN-β has been shown to increase NK activity to a higher level than that reached with either of the cytokines alone (20). Likewise, a combination of rIFN-γ with IL2 resulted in an increase in NK cytotoxicity which was more than additive (21, 22).

Our recent in vivo study reported the combination of human rHIL2 and murine rIFN-β to produce a marked regression of s.c. established adenocarcinoma 755 in C57BL/6 mice, which rHIL2 or rIFN-β alone did not. The marked regression of s.c. adenocarcinoma 755 by rHIL2 and rIFN-β therapy was not observed in athymic (nude) mice (23). These studies indicate T-cell immunity to be important in the regression of the s.c. established adenocarcinoma 755.

Further information is needed on the extent to which rHIL2 and rIFN combinations show the antitumor effect in various tumor-bearing mice, and these studies investigating antitumor activities in rHIL2-rIFN combinations will prove extremely important preclinically.

MATERIALS AND METHODS

Mice. Inbred 5-wk-old male C57BL/6 mice and BALB/c mice were obtained from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Male beige mice (C57BL/6 background) were supplied from Japan Charles River Co., Ltd. (Atsugi, Japan). These were maintained under specific-pathogen-free conditions in our laboratory. All experiments were initiated when the mice were 7 wk old.

Tumor. Adenocarcinoma 755 (20 mg/mouse), B16-F10 melanoma (4 x 10⁵ cells/mouse), Lewis lung carcinoma (4 x 10⁵ cells/mouse), colon 38 (20 mg/mouse), and colon 26 (1 x 10⁶ cells/mouse) were implanted s.c. into the right hind legs of the mice, causing a s.c. tumor nodule to appear at the inoculation site in all animals between Days 5 and 12. All mice used in a single experiment (6 to 8 mice/group) were inoculated on the same day using cells of the same suspension.

Drugs and Treatments. Lyophilized rHIL2 (specific activity, 1 x 10⁷ units/mg protein) was kindly supplied by Biogen S. A. Company, Switzerland, and Shionogi & Co., Ltd., Osaka, Japan. The rIFN-α A/D was generously provided by Nippon Roche, Kamakura, Japan (specific activity, 2.04 x 10⁷ IU/ml). The rIFN-β (specific activity, 5.5 x 10⁵ IU/mg protein) and rIFN-γ (specific activity, 5 x 10⁶ units/mg protein) were supplied by Toray Industries, Inc., Kamakura, Japan.

When the tumors became palpable (3-5 mm diameter), rHIL2 and the rIFNs were administered s.c. at the other site (left thigh) of tumor implantation, and this was continued daily for a period of 8 or more days. The tumor diameters were measured at the longest (a) and shortest (b) arms 3 times each week using calipers, and the volume was calculated using the formula: a²b/2 (mm³).

Cytotoxicity Assay. To determine cell-mediated cytotoxicity, Na₂¹⁴C³O₂ (Amersham, England)-labeled target cells (YAC-1 and B16-F10 melanoma) were incubated with spleen cells of mice (C57BL/6)
treated 8 times with an injection of rHIL2 at 10⁶ units/mouse and/or rIFN-β at 10⁵ IU/mouse in Linbro 96-well microtiter plates (Linbro Scientific Co., McLean, VA). Details of the technique have been described previously (24).

Statistics. Experimental data were statistically evaluated using the two-sided t test.

RESULTS

Therapeutic Effects of rHIL2 and/or the rIFNs in Adenocarcinoma 755-bearing Mice. C57BL/6 mice inoculated s.c. (right) with adenocarcinoma 755 were s.c. administered (left) with various doses of rHIL2 and/or rIFN-β for 8 days starting 8 days after tumor inoculation. The antitumor effect was measured by tumor size at different intervals (Fig. 1). At 10⁵ IU of IFN-β alone, tumor growth was hardly suppressed, whereas a moderately suppressive effect on tumor growth by rHIL2 was seen at a dose of 10⁶ units. The combination of 10⁶ units of rHIL2 and 10⁵ IU of rIFN-β produced a more marked regression of s.c. established tumor than did rHIL2 alone, but the combination of 10⁵ units of rHIL2 and 10⁴ IU of rIFN-β was as effective as rHIL2 alone. Moreover, all mice (6 of 6) showed not only tumor growth retardation but apparently, experienced a complete cure (tumor free for 3 mo) in the group which received the combination of rHIL2 at 10⁶ units and rIFN-β at 10⁵ IU for longer periods (13 injections), although rHIL2 or rIFN-β alone produced no regression (Fig. 2A). One of the antitumor agents, 5FU at a maximum nontoxic dose of 20 mg/kg, i.v., which had an antitumor effect on early tumors (25), hardly inhibited the growth of the established tumor (Fig. 2A). Upon further delayed treatment, starting 11 days after tumor inoculation (tumor size, 1 to 2 cm³), complete tumor regression (7 of 8) was shown after 18 injections (Fig. 2B). When the tumor-free mice were rechallenged with the tumor 60 days after complete regression, the tumor was completely rejected.

Furthermore, rIFN-α A/D or rIFN-γ was used for treating the s.c. established adenocarcinoma 755 under a similar regimen as that used in the rHIL2-rIFN-β combination. The combination of rIFN-α A/D (10⁴ IU/mouse) and rHIL2 (10⁶ units/mouse) produced a marked regression of the established tumor, and some mice (3 of 8) were cured (Fig. 3). On the other hand, the administration of rHIL2 and rIFN-γ (10⁵ and 2 × 10⁴ units/mouse) resulted in a retardation at most, not a regression (Fig. 4). All mice showed a regrowth a few days after the treatment had been stopped. Thus, the combination of rHIL2 and rIFN-α A/D or -β gave better results than the sole administration of either or the administration of rHIL2 and rIFN-γ in combination.

Therapeutic Effects of rHIL2 and/or the rIFNs in B16-F10 Melanoma-, Colon 38-, Lewis Lung Carcinoma, and Colon 26-bearing Mice. Subsequent experiments were performed to examine the antitumor effects, if any, of rHIL2 and the rIFNs in different tumor systems. The treatments were begun on Day 5 postimplantation, and comprised 13 and 10 injections, respectively, of rHIL2 and the rIFNs against B16-F10 melanoma and Lewis lung carcinoma in C57BL/6 mice. The B16-F10 melanoma responded to the combination of rHIL2 (10⁶ units/mouse) and rIFN-α A/D (10⁵ IU/mouse) to a similar extent as did the adenocarcinoma 755. The combination of rHIL2 and rIFN-β (10⁵ IU/mouse) also retarded tumor growth, but a
On the other hand, no combination of rHIL2 and the rIFNs was effective, or was only marginally so, if at all, against the Lewis lung carcinoma (Table 2).

In the colon 38 system in C57BL/6 mice, treatments of rHIL2 and rIFNs began on Day 12 postinoculation and comprised 13 injections. The combination of rHIL2 and rIFN-α A/D produced a marked regression of the tumor, and some mice (4 of 6) were cured completely (Fig. 5), although rHIL2, or rIFN-α A/D alone, was not effective or had some retardation effect, respectively. Moreover, the combination of rHIL2 with rIFN-β produced a marked retardation of tumor growth, although rHIL2 or rIFN-β alone was not effective at all.

In another experiment, the antitumor effects of rHIL2 and the rIFNs were examined in a different strain of mouse. Under a similar regimen to that used in the adenocarcinoma 755 system (C57BL/6), colon 26 in BALB/c mice responded to the rHIL2-rIFN-α A/D or -β combination therapy to a similar extent as did the adenocarcinoma 755. The rHIL2-rIFN-α A/D or -β combination retarded tumor growth and significantly increased the life span by 75 or 106%, respectively (Table 3).

Thus, rHIL2 therapy in combination with rIFN-α A/D or -β produced a marked potentiation of the antitumor effect in many murine tumor systems without increasing toxicity to the host.

Role of NK Cells in the rHIL2-rIFN-β Combination in the Regression of Adenocarcinoma 755. In the study with nude mice (23), the synergistic effect of rHIL2-rIFN-β was completely nullified, suggesting the T-cell-associated immunity is involved in this therapy. rHIL2 and the rIFNs are known to enhance NK activity, however (11-16). Various recent reports suggest that NK cells have an important role to play in resistance to tumor development in vivo (26). The activation of NK cells may, therefore, be involved in the tumor's regression. In order to evaluate the role of NK cells in tumor regression, we examined the effectiveness of the rHIL2-rIFN-β combination against adenocarcinoma 755 in C57BL/6 beige mice (bg/bg).

Beige mice bearing adenocarcinoma 755 were administered with rHIL2 and/or rIFN-β, and their tumor sizes were measured after treatment (Fig. 6). The combinations produced regressions in tumor growth even in beige mice; 4 of 6 mice given the rHIL2-rIFN-β combination were cured. Moreover, in C57BL/6 mice bearing adenocarcinoma 755, this combination therapy is not affected by treatment with the antiasialo-GM1 antibody (23). And, in vitro study, cytotoxic activity against YAC-1 and B16-F10 melanoma cells was enhanced by the rHIL2-rIFN-β combination and was almost similar to that by rHIL2 alone (Table 4). These results suggest that activated host NK cells were not responsible for the therapeutic effect against adenocarcinoma 755.

DISCUSSION

The present study has shown the combination therapies of rHIL2 and either rIFN-α A/D or -β to be markedly effective in

combination with rIFN-β (2 x 10⁴ units/mouse) had hardly any inhibitory effect after 13 injections. As a result of it, the rHIL2-rIFN-α A/D or -β combination significantly increased the life span by 57 or 46%, respectively (Table 1).
ANTITUMOR ACTIVITY OF rHIL2 AND rIFNs

Fig. 5. Effect of rHIL2 (10^6 units/mouse) and/or rIFNs (10^6 IU/mouse) on tumor growth of colon 38 in C57BL/6 mice. J, s.c. treatment. It was decided which mice were tumor free 4 mo after tumor inoculation. Control, O; rHIL2, •; rIFN-α A/D, △; rIFN-α A/D + rHIL2, □; rIFN-β, ▲; rIFN-β + rHIL2, △.

Table 3 Antitumor effect of rHIL2 and rIFNs against colon 26 in BALB/c mice

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Control</th>
<th>rIFN-α A/D, 10^6 IU/mouse*</th>
<th>rIFN-β, 10^6 IU/mouse</th>
<th>rIFN-γ, 2 × 10^6 units/mouse</th>
<th>rIFN-γ, 10^6 units/mouse</th>
<th>rHIL2, 10^6 units/mouse</th>
<th>rHIL2 + rIFN-α A/D</th>
<th>rHIL2 + rIFN-β, 2 × 10^6 units/mouse</th>
<th>rHIL2 + rIFN-γ, 10^6 units/mouse</th>
<th>rHIL2 + rIFN-β</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>18</td>
<td>15.9 ± 0.3</td>
<td>20.1 ± 2.0</td>
<td>19.5 ± 1.5</td>
<td>15.1 ± 0.4</td>
<td>18.1 ± 1.6</td>
<td>19.2 ± 1.2</td>
<td>27.9 ± 1.3</td>
<td>19.4 ± 1.4</td>
<td>75± 2.3</td>
</tr>
</tbody>
</table>

* Mice were given injections s.c. on Days 7 to 14.
* P < 0.01 compared to rIFN-α A/D or rHIL2 alone.
* P < 0.001 compared to rIFN-β or rHIL2 alone.

Table 4 Augmentation of killing activity of spleen lymphocytes by rHIL2 and rIFN-β in tumor-bearing mice (adenocarcinoma 755)

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>YAC-1</th>
<th>B16-F10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-tumor-bearing mice</td>
<td>6.1 ± 2.0</td>
<td>0</td>
</tr>
<tr>
<td>Tumor-bearing mice (control)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rIFN-β, 10^6 IU/mouse</td>
<td>8.8 ± 0.2</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>rHIL2, 10^6 units/mouse</td>
<td>36.3 ± 4.1</td>
<td>14.8 ± 1.5</td>
</tr>
<tr>
<td>rHIL2 + rIFN-β</td>
<td>34.2 ± 2.2</td>
<td>17.4 ± 0.5</td>
</tr>
</tbody>
</table>

* Cytotoxicity against target cells was determined on Day 15 after 8 injections of rHIL2 and/or rIFN-β (Days 7 to 14). Three mice/group were used.
* Murine spleen cells (1 x 10^6/well) were assayed for cytotoxicity against target cells (1 x 10^6/well).
* Mean ± SE.

mice inoculated s.c. with solid tumors: adenocarcinoma 755, B16-F10 melanoma; and colons 26 and 38. These tumors were insensitive to treatment by either rHIL2 or the rIFNs unless they were combined. Adenocarcinoma 755- and colon 38-bearing mice, especially, were cured by the rHIL2 plus rIFN-α A/D or -β treatments. The potentiation of in vivo antitumor activity in the combinations of rHIL2 and the rIFNs was analyzed from the points of view of inhibition of tumor growth and the host-mediated activity. The tumors which regressed after the rHIL2-rIFN-β treatment became immune to adenocarcinoma 755, eventually resisting the s.c. challenge of 5 x 10^6 adenocarcinoma 755 cells. It has been shown that the immune T-cells produced by rHIL2-rIFN-β combination therapy were able to be retained and that they still possessed in vivo activity against tumor challenge. Moreover, in nude mice, the therapeutic effect, if any, of the rHIL2-rIFN-β combination was marginal and much less effective than in T-cell-competent (+/++) mice (23). Thus, treatment with rHIL2 alone or rIFN alone had no impact on the survival of tumor-bearing mice, but the combination of rHIL2 and rIFN-α A/D or -β improved their survival and resulted in cure in some T-cell-competent mice.

IL2 is essential for the generation of CTL. The administration of IL2 in vivo has been shown to induce the growth of antigen-activated T-lymphocytes (27). The in vivo treatment with the interferon inducer, copolymer of polyinosinic and polycytidylic acids, or the in vitro incubation of IFN-α/β enhances the expression of lymphocyte differentiation antigens on murine T-cells, especially resting T-cells (28). The enhanced expression following IFN-IL2 treatment could be the cause of the activation process in the T-cells. Alternatively, IFN may act as a CTL differentiation signal, although the mechanism by
ACKNOWLEDGMENTS

These studies thus strongly suggest rHIL2 and rIFN-α or -β to be of value as therapeutic agents for cancer.

ANTITUMOR ACTIVITY OF rHIL2 AND rIFNs

which T-lymphocytes in the presence of IFN acquire antigen-specific cytotoxic function still remains unclear. IFNs have been shown to augment NK activity in vitro and in vivo (11-13). The in vivo administration of IFNs can cause an earlier maturation of NK activity. NK cells have been shown to mediate cytotoxic effects against a variety of tumor cells in vitro, and this activity is augmented by the in vivo administration of IFN, IFN-inducing Biological Response Modifiers, and various other cytokines. Moreover, when combined with IL2, IFN-β has been shown to increase NK activity to a higher level than that reached with either of the cytokines alone (20). In this experiment, adenocarcinoma 755 was markedly regressed by the combination of rHIL2 and rIFN-β in beige mice which have low NK activity. Moreover, this combination therapy is not affected by treatment with the anti-asialo-GM1 antibody (23). Thus, the therapeutic activation of rHIL2 plus rIFN-β occurred despite NK hyporesponsiveness. The combination of rHIL2 and rIFN-β produced enhanced NK activity, but it was almost the same as that by rHIL2 alone.

The potentiation of antitumor activity was obtained by the combination of rHIL2 with rIFN-α A/D or -β, but not with rIFN-γ. IFN-α (or -β) and IFN-γ are known to have distinct receptors (29). These results suggest that the receptor of IFN-α or -β is very important.

In conclusion, combination therapy with rHIL2 and IFN-α A/D, which is similar to natural murine IFN-α (30, 31) or -β, very important. rHIL2 alone.

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