ABSTRACT

A highly purified hybrid human interferon (IFN)-α A/D, was used to define optimum treatment regimens for Meth A fibrosarcoma intradermally inoculated in syngeneic BALB/c mice. Treatments from the sixth day post-tumor inoculation were most effective, and 10 consecutive injections of IFN completely suppressed the tumor growth for a prolonged time without recurrence after the last injection of IFN. Intraperitoneal and intravenous injections were similarly effective.

The therapeutic effect of IFN was abrogated by the injection of rabbit α-mouse thymocyte globulin but not normal rabbit globulin. The therapeutic effect of IFN was much poorer in BALB/c nu/nu mice, which are athymic and defective in T-cell immunity, than in BALB/c +/+ mice. Mice in which Meth A growth was completely suppressed by IFN were refractory to Meth A tumor, but not Meth 1 tumor, another antigenically distinct fibrosarcoma, whereas mice in which Meth 1 growth was suppressed completely by IFN were refractory to Meth 1 but not Meth A tumor. These three findings suggest that the therapeutic effect of IFN depended on host T-cell immunity and that tumors were eventually eliminated by tumor-selective antitumor immunity provoked during IFN therapy. Under the present experimental conditions, the direct antitumor effect of IFN was presumed to contribute very little to the therapy for solid tumors, since the therapeutic effect of IFN on three different solid tumors was not correlated with their in vitro sensitivity to this substance: Colon 26, which was as sensitive to IFN as Meth A in the in vitro antiproliferation, responded most poorly to IFN therapy, while Meth A and Meth 1 responded similarly well to IFN therapy, although there was more than a 2500 times difference in their in vitro IFN sensitivity.

INTRODUCTION

There is now a considerable amount of data on the variety of effects of IFN-α,β on tumor cells and host immunocytes (1). However, it has yet to be proven whether any of these findings were associated with the in vivo therapeutic effect of IFN. In solid tumors, an alternative hypothesis was recently proposed in nonviral tumor models, especially nonimmunological ways (2). However, it has yet to be proven whether any of these findings were associated with the in vivo therapeutic effect of IFN. In solid tumors, the therapeutic effect of IFN was strongly associated with the in vivo therapeutic effect of IFN. In this regard, we selected a methylcholanthrene-induced fibrosarcoma, Meth A, and showed previously that mouse L-cell-derived IFN suppressed the growth of i.d. inoculated Meth A fibrosarcoma in the host T-cell-dependent fashion (3). However, a limited supply and low specific activity of the IFN preparation did not warrant the further characterization of its antitumor activity.

Recent development of gene technology provided us with a novel molecular hybrid IFN of human origin, IFN-α A/D, which was strongly reactive with mouse cells (4). We found that, under specified conditions, IFN-α A/D cured Meth A-bearing syngeneic BALB/c mice and that various aspects of its therapeutic activity, i.e., administration routes, tumor panels, and host T-cell dependency, were basically similar to those obtained with mouse L-cell-derived IFN (3). These results warrant the further characterization of host-dependent mechanisms of antitumor activity of IFN in solid tumor models by the use of human IFN-α A/D.

MATERIALS AND METHODS

Mice and Tumors. Female BALB/c (+/+ and nu/nu) mice were obtained from Japan Charles River Co., Ltd (Atsugi, Kanagawa, Japan). Meth A fibrosarcoma was supplied by Dr. Y. Hashimoto. Meth 1 fibrosarcoma, furnished in ascitic form by Dr. M. Morimoto, was subcutaneously passaged in our laboratory, and its transplantable fraction was selected. Colon 26 carcinoma was from the National Cancer Institute (Bethesda, MD).

Interferon. INF-α A/D (Bgl) was purified from Escherichia coli W3110/pLIF-a A/D (Bgl) cells by a combination of methods described previously (4). The purified material was homogeneous by polyacrylamide gel electrophoresis and had a specific activity of more than 10^6 units/mg protein when titrated on bovine kidney cells (MDBK). Antiviral activity was titrated against the NIH standard (Gxa 01-901-535). IFN was supplied in 0.9% NaCl solution containing IFN (100 μg/ml) and mouse serum albumin (1 mg/ml), Fraction 5, prepared from ddy mice. Placebo contained the same ingredients as above, except for IFN, which was omitted.

In Vitro Antiproliferation Test. Tumor cells were cultivated in Roswell Park Memorial Institute 1640 medium [Grand Island Biological Co. (GIBCO), Grand Island, NY] containing 10% fetal bovine serum (GIBCO), kanamycin (100 μg/ml), (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) and 20 μM mercaptoethanol (Wako Pure Chemical Industries, Ltd., Tokyo) in the presence of different concentrations of IFN in a CO2 incubator (WJ-22-C, Hirasawa Works, Tokyo) at 37°C for 4 or 5 days. Meth A, Meth 1, and Colon 26 cells were cultivated in a 24-well tissue culture flask (Nunc Multidish, Denmark). After cultivation, Meth A and Meth 1 cells were diluted in Isoton (Coulter Electronics, Inc., Hialeah, FA) and counted by a Coulter counter (Model ZB, Coulter Electronics, Inc.). Colon 26 cells were first incubated with 0.05% trypsin-0.02% EDTA (GIBCO) at 37°C and were then mixed with an equivalent volume of 0.85% NaCl solution containing 10% fetal bovine serum. Single-cell suspensions were prepared by pipetting, were diluted in Isoton, and were counted by a Coulter counter. In cell counting, a hemocytometer (Model 4000, Clay Adams Division of Becton, Dickinson and Co., Parsippany, New Jersey) was used to count cells.
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NJ) and a Coulter counter gave essentially the same results (<10% difference).

Therapy by IFN. Groups of mice inoculated i.d. with 2 x 10^6 tumor cells (1 x 10^6 in nu/nu mice) were administered IFN in 0.20 ml of 5 mm phosphate-buffered saline solution (pH 7.2) containing 0.85% NaCl/mouse/day. Under the present experimental conditions, administration of placebo neither enhanced nor suppressed the tumor growth. Tumor sizes were measured at the longest (a) and shortest (b) arms and expressed as √ab. In some experiments, therapeutic effect was expressed as a percentage of inhibition of control tumor growth according to the following formula:

\[
\left(1 - \frac{\text{Tumor size of mice treated with IFN}}{\text{Tumor size of control (nontreated) mice}}\right) \times 100
\]

In Vivo Abrogation of T-Cells. Rabbit α-mouse thymocyte globulin and rabbit normal globulin were obtained from Microbiological Associates (Bethesda, MD). The in vivo potency of rabbit α-mouse thymocyte globulin in abrogating T-cell immunity was established by the supplier in prolonging the survival of allografted skin and also by us in nullifying antitumor T-cell immunity in mice hyperimmunized to L1210 leukemia (5). We further confirmed the selective elimination of T-cells by rabbit α-mouse thymocyte globulin in the spleen and blood of BALB/c mice (3): 3 days after inoculation of 0.25 ml of rabbit α-mouse thymocyte globulin, spleen T-cells but not B-cells were decreased to 36% of control spleen T-cells, and T-cell content in peripheral blood dropped to one-third of that of control blood. Normal rabbit globulin did not cause any change of T-cell population in spleen and blood under these experimental conditions.

Statistics. Tumor sizes of mice in the 2 groups were statistically evaluated by the f-test. Incidences of tumor-free mice were evaluated by the Fisher exact test. In some experiments, percentage of inhibition values of tumor sizes of Meth 1 and Colon 26 in +/+ mice or Meth A in nu/nu mice were compared with those of Meth A tumor in +/+ mice. The percentage of inhibition value of a single experiment was evaluated by Thompson's interval estimate and considered significant when it was beyond the values of 95% confidence limits of mean values of Meth A estimated from the results of 7 Meth A experiments.

RESULTS

Administration Intervals of IFN. BALB/c mice that had been inoculated i.d. with Meth A cells at Day 0 were administered IFN i.p. once per day for 10 days starting at different intervals (Chart 1). Delayed administration of IFN (Days 6 to 15) produced more beneficial effect than did its earlier administration (Days 1 to 10) and completely suppressed the tumor growth for a prolonged period of time in many mice (observation period, 49 days in Experiment 1 and 39 days in Experiment 2). These mice were considered cured because of resistance to reinoculation of Meth A cells. This will be evidenced later.

The optimal administration intervals were more critically evaluated under the conditions in which IFN was administered for shorter intervals (5 consecutive days) (Chart 2). IFN administered at Days 6 to 10 produced more beneficial effects than that administered at earlier or later intervals (Days 1 to 5 or Days 11 to 15). The results confirmed the above findings (Chart 1), showing that the earlier administration did not necessarily produce a more beneficial effect.

Administration Route and Dose of IFN. We compared the therapeutic effect of i.p. and i.v. administration of IFN of higher doses (2 x 10^6 units/day, 6 days) (Chart 3). We found both i.p. and i.v. administration of IFN effective, confirming the results obtained with mouse IFN (3). For the rest of the study, IFN was i.p. administered.

The therapeutic effect of IFN was dose-dependent (Chart 4) and, under the regimen of Day 5 to 10 administration, IFN (6.3 x 10^4 units/day) produced the significant inhibition of tumor growth, and higher doses produced more beneficial effects, ultimately curing some mice at 0.25 and 1.0 x 10^6 units/day.

T-Cell Involvement in the IFN-dependent Therapeutic Effect. We showed previously that the therapeutic effect of mouse IFN to Meth A was host T-cell-dependent (3). We tested whether this was the case with IFN-α A/D. I.p. administration of rabbit α-mouse thymocyte globulin but not rabbit normal globulin abrogated the IFN-dependent therapeutic effect (Chart 5), suggesting that T-cell-associated immunity was involved in the IFN-dependent therapeutic effect. We extended this hypothesis and examined whether IFN was effective in Meth A-bearing BALB/c nu/nu...
mice, which are athymic and defective in T-cell-mediated immunity. IFN produced a marginal effect, if any at all (Chart 6), and the suppression of tumor growth in these nu/nu mice (maximal inhibition percentage of control tumor size during the observation period, 21% in Experiment 1 and 26% in Experiment 2; Chart 6) was smaller (significant at $P < 0.05$ by the Thompson's interval estimate) than that achieved in +/+ mice (mean ± SD of 7 experiments, 44.5 ± 9.4). Furthermore, tumors of the IFN group, although delayed, grew progressively at the same rate as did that of the control group. This is in sharp contrast to the results in +/+ mice (Charts 1 to 5). These results do not eliminate the contribution of the direct antiproliferative effect of IFN on the therapeutic effect but further indicate the dependence of the therapeutic effect of IFN on the host T-cell immunity.

Tumor Selective Antitumor Activity in Tumor-bearing Mice Treated with IFN. Mice i.d. inoculated with Meth A or Meth 1 cells at Day 0 were administered IFN i.p. (10^6 units) once a day for 10 days starting at Day 5 or later. At intervals of more than 1 week after the last injection of IFN, each of those mice in which tumor nodules were not progressing during the observation period was reinoculated i.d. with both Meth A and Meth 1 cells (2 x 10^5 cells) separately on either side of the flank and examined for tumor growth (Table 1). These 2 tumors are antigenically distinct fibrosarcomas (6). In Meth A-cured mice, the growth of
Meth A was almost completely suppressed, whereas that of Meth 1 in the same individuals was not modified as compared with that in the control mice in terms of tumor incidence and tumor size (Experiments 1 and 2). In Meth 1-cured mice, the growth of Meth 1 but not Meth A was completely suppressed (Experiment 3). These results not only indicate that IFN cured tumor-bearing mice but also extend the earlier observation of host T-cell dependence of IFN antitumor activity to suggest that IFN administration resulted in provoking the tumor selective antitumor effect in T-cell-dependent fashion.

Therapeutic Effect of IFN in Different Tumors and Their In Vitro Sensitivity to IFN. The therapeutic effect of IFN was compared in another 2 solid tumors inoculated i.d. in syngenic BALB/c mice under a regimen similar to that used in the Meth A experiments (Table 2). IFN was effective for Meth 1 to a similar extent as for Meth A. There was no statistical difference in the maximal inhibition percentage of control tumor size between the 2 tumor models (41 and 49% of Meth A versus 44.5 ± 9.4 of 7 Meth A experiments).

However, IFN was less effective for Colon 26 than for Meth A under the regimen optimal to Meth A (Colon 26, Experiment 1). Statistically significant at $P < 0.05$ by Thompson's interval estimate versus 44.5 ± 9.4% inhibition of Meth A). Since it was presumed that IFN administration at Days 5 to 10 post-tumor inoculation might not be optimal for Colon 26, IFN was adminis-
tered at Days 12 to 19 (Experiment 2). Higher tumor size inhibition, 31%, was achieved. However, Colon 26 grew progressively under both IFN regimens, whereas Meth 1 was stable or regressing, as was the case with Meth A. It was also noted that there was no difference in the suppression of Colon 26 growth by IFN at the doses studies. This was in sharp contrast with the dose-dependent effect of IFN for Meth A tumors.

To characterize these differences of therapeutic effect in the 3 tumors, we examined the in vitro sensitivity of tumor cells to the direct antcellular activity of IFN (Table 3). Colon 26 and Meth A cells were similarly sensitive to IFN, whereas Meth 1 cells were quite resistant to IFN and were suppressed in proliferation only marginally by IFN, even at $6.4 \times 10^8$ units/ml. These results suggest that the difference of the in vivo therapeutic effect of IFN in these tumors was not substantiated by their sensitivity to the antiproliferative activity of IFN and point to the involvement of host-mediated response in the therapeutic effect of IFN.

**DISCUSSION**

The present study demonstrated that the therapeutic effect of IFN-α A/D to Meth A was dependent on the administration intervals and dose but not much on the administration route. Meth A-bearing mice were cured only when IFN administration started with delayed timing (6 days after tumor inoculation) and continued for an extended period (10 days). Additionally, we noted that administration every other day was less effective than that every day (data not presented). These results are not consistent with those obtained with IFN-α A/D in L1210 leukemia, in which the efficacy was greatest with administration every second or third day, but not every day, commencing one day after tumor inoculation (7). We note the following differences of experimental conditions between the two animal models: tumor types, solid fibrosarcoma versus ascitic leukemia; mouse strain, syngeneic BALB/c versus histoincompatible B6D2F1. We do not know whether any of these differences was associated with the different optimum treatment regimens.

We showed that the therapeutic effect of IFN in Meth A was dependent on the host T-cell immunity, confirming the previous results with mouse IFN (3). Furthermore, mice in which tumor growth was suppressed by IFN for a prolonged period of time were refractory to the same tumor but not to the antigenically distinct tumor, indicating that IFN therapy induced tumor-selective antitumor immunity. These results are in contrast with the
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Table 2
Antitumor activity of IFN in Meth 1 and Colon 26-bearing mice

Mice that had been inoculated i.d. with 2 x 10^6 cells of Meth 1 or Colon 26 were administered IFN i.p. according to the following regimens: Meth 1, 1 x 10^6 units/ide/mouse (12 mice, Experiment 1) or 2 x 10^6 units/10 mice, Experiment 2) at Day 6 to 11 post-tumor inoculation; Colon 26, 2 x 10^6 to 0.3 x 10^6 units at Day 5 to 10 (18 mice, Experiment 1) or 10^6 units at Day 12 to 19 (6 mice, Experiment 2) post-tumor inoculation. They were compared for tumor sizes at 2 different intervals to examine whether the tumors were progressing, stable, or regressing. In Colon 26 (Experiment 1), since we did not find any difference in tumor size among the IFN groups at 3 different doses as indicated in the text, these mice were classified into one group.

Table 3
In vitro antiproliferative activity of IFN in 3 murine tumor cells

Tumor cells (2 x 10^6/ml) were incubated in vitro with different concentrations of IFN for 4 or 5 days. The cell concentrations were measured by a Coulter counter.

Table 4

<table>
<thead>
<tr>
<th>Tumor growth* in mice treated with:</th>
<th>% inhibition of control tumor growth at the termination of experiment</th>
<th>Tumor state of IFN-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice bearing:</td>
<td>Experiment no.</td>
<td>Nothing</td>
</tr>
<tr>
<td>Meth 1</td>
<td>1</td>
<td>13.5 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>Colon 26</td>
<td>1</td>
<td>14.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.2 ± 1.3</td>
</tr>
</tbody>
</table>

* Expressed as mean of tumor sizes at the termination of experiments (>Day 18) and about 1 week before the termination (in parentheses).

The present study not only showed the efficacy of IFN for solid tumors but also pointed out that IFN might not be effective for other solid tumors. We do not know what is responsible for this difference. The immunogenicity of tumor cells may be one of the factors. In fact, Meth A and Meth 1 tumors were immunogenic, and their vaccine cells induced tumor-selective antitumor immunity in BALB/c mice (6). We need to compare the immunogenicity of Colon 26, a poor responder to IFN, and that of Meth A and Meth 1 under the same experimental conditions. It was reported in Meth A that IFN increased the expression of H-2 antigen, whereas it decreased that of tumor-associated antigen (11). Since both of these antigens are associated with tumor rejection by host T-cells, a comparative analysis of Meth A and Colon 26 in the change of antigen expression by IFN may provide significant information in this regard.

It was also noted that the therapeutic effect of IFN on Colon 26 was marginal and that no differences were noted at the IFN doses studied. This may be a reflection of how important an IFN-antiproliferative contribution of IFN to the therapeutic effect. In fact, IFN was marginally but definitely effective for Meth A in nu/nu mice. However, the finding that IFN administration at delayed intervals (Days 6 to 15 post-tumor inoculation) produced cured mice at a much higher incidence (Chart 1, Experiments 1 and 2; number of cured mice/total, 8/16) than that administered at earlier intervals (Days 1 to 10; 1/16) does not warrant the antitumoral activity of IFN in terms of the antitumoral activity of IFN as being the major factor in the therapeutic effect under the present experimental conditions.

ACKNOWLEDGMENTS

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