Relative Contribution of Antiproliferative and Host Immunity-associated Activity of Mouse Interferon in Murine Tumor Therapy

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ABSTRACT

Administration of mouse interferon (IFN; 0.5 to 1 x 10^7 reference units/mg protein) inhibited the growth of Meth A and Meth 1 fibrosarcomas, but to a lesser extent, if at all, the growth of Colon 26 adenocarcinoma in BALB/c mice. The in vitro IFN sensitivity of these three tumors was not consistent with the in vivo therapeutic response in mice bearing these tumors under the present experimental conditions; Colon 26, the most sensitive of the three tumors in the in vitro antiproliferation test, did not respond or responded most poorly to IFN therapy; furthermore, Meth A and Meth 1 tumors responded similarly well to IFN therapy, although there was about a 100-fold difference in their in vitro IFN sensitivity. These results as well as the kinetic analysis of IFN concentrations of the serum of Meth A- or Colon 26-bearing mice did not indicate that the antiproliferative activity of IFN was solely responsible for its in vivo therapeutic effect.

In contrast, abrogation of T-cell immunity by a mouse thymocyte globulin completely nullified the IFN-dependent therapeutic effect in Meth A-bearing mice. Furthermore, the IFN-dependent therapeutic response in Meth A tumors was much weaker in T-cell-defective BALB/c (nu/nu) mice than that in immunologically competent BALB/c (+/+)) mice and was marginal, if present at all, confirming that T-cell immunity was involved in the IFN-dependent therapeutic effect and suggesting that the antiproliferative activity of IFN may only be responsible to a small extent for the therapeutic effect.

INTRODUCTION

There is now a considerable amount of data on the variety of effects of IFN on tumor cells and host immunocytes (13). However, it is yet to be proven whether any of these findings were associated with the in vivo therapeutic effect of IFN. The accumulation of experimental findings, such as the in vitro antiproliferative effect of IFN on human tumor cells and the therapeutic effect of IFN in immunologically defective athymic mice bearing these tumors, may not necessarily indicate that the antiproliferative activity of IFN plays a major role in its therapeutic effect in a tumor-bearing host equipped with a normal immunological apparatus.

On the other hand, the hypothesis that the antitumor activity of IFN is host-mediated was largely based on the finding that IFN was therapeutically effective in mice bearing IFN-resistant as well as IFN-sensitive tumors (2, 6). Although these studies clearly demonstrated that the IFN-dependent therapeutic effect was host-mediated, they did not identify the host antitumor immunity as the host mediator. A recent study reported that there was no difference in host immunocyte infiltration between the tumor nodules that progressed in untreated mice and those that regressed in IFN-treated mice. Based on these findings, it was proposed that the IFN-dependent elimination of solid tumors was due to mechanisms that have not been seriously considered heretofore (1).

At this moment, we need further information on how much the antiproliferative and/or host-mediated activity of IFN was involved in the therapeutic effect in tumor-bearing mice and also whether the host immunity was identified as an IFN-dependent host mediator in the therapeutic response. The present study indicates that, under the present experimental conditions, T-cell immunity was involved in the IFN-dependent therapeutic effect and that the antiproliferative activity of IFN contributed only to a small extent to the therapeutic response.

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, supplied in ascitic form by Dr. M. Morimoto, was passaged s.c. in our laboratory, and its transplantable fraction was selected. Colon 26 adenocarcinoma was supplied by the National Cancer Institute (Bethesda, MD).

IFN. Mouse IFN (a,ß) was prepared as follows. L-MS cells, the cloned mouse L-cells producing IFN at a high efficiency, were washed in Eagle’s minimal essential medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) and incubated with Newcastle disease virus. After washing, the cells were incubated further, and their culture fluid was acidified to pH 2 and subsequently neutralized. It was concentrated and dialyzed in a cellulose tube (Sanko Pure Chemical Industries, Tokyo, Japan) and then kept frozen at -20°. The antiviral activity was assayed by the cytopathic effect of vesicular stomatitis virus on mouse L-cells and expressed in units with reference to the NIH standard (G002-904-511). Protein concentrations were estimated by using bovine serum albumin, Fraction 5 (Sigma Chemical Co., St. Louis, MO) as a standard (9). The specific activity of IFN was 0.5 to 1 x 10^7 units/mg protein.

In Vitro Antiproliferation Test. Tumor cells were cultivated in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum (Grand Island Biological Co.), 100 µg of kanamycin per ml (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), and 20 µM mercaptoethanol (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) in the presence of different concentrations of IFN in a CO _2 incubator (WU-22-C; Hirasawa Works, Tokyo, Japan) at 37° for 4 days. Meth A and Meth 1 cells were cultivated in glass test tubes. Colon 26 cells were cultivated in a 24-well tissue culture flask (Nunc multidiish; Nunc, Roskilde, Denmark). After a 4-day cultivation, Meth A and Meth 1 cells were diluted in isoton (Coulter Electronics Inc., Hialeah, FL) and counted by a Coulter Counter (Model ZF; Coulter). Colon 26 cells were first incubated...
with 0.05% trypsin:0.02% EDTA (Grand Island Biological Co.) at 37° and then mixed with an equivalent volume of 0.85% NaCl solution containing 10% fetal bovine serum. Single-cell suspensions were prepared by pipetting, diluted in Isoton, and counted by a Coulter Counter.

Therapeutic Regimen of IFN. Groups of 6 or 7 mice inoculated i.d. with tumor cells (10⁵ of Colon 26, 2 x 10⁴ of Meth A, 1 x 10⁵ of Meth 1) were given (except as shown in Chart 1) 2 x 10⁶ units of IFN in 0.20 ml of 0.85% NaCl solution/shot/mouse/day i.v. for 10 consecutive days starting 1 day after tumor inoculation, except as otherwise stated. Tumor sizes were measured at the longest (a) and shortest (b) arms and expressed as √ab.

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 (8). We further examined the elimination of T-cells by rabbit α mouse thymocyte globulin in the spleen and blood of BALB/c mice. A single cell suspension of spleen was prepared according to the method reported previously (8). Blood was collected by cardiac puncture from anesthetized mice and centrifuged onto lymphocyte-M solution (Cedarslane Laboratories, Ltd., Ontario, Canada) at 500 x g for 20 min, and the leukocyte fraction was collected. After hemolysis, spleen and peripheral lymphocytes (1 x 10⁷/ml) were fixed with 1% paraformaldehyde (E. Merck, Darmstadt, West Germany) at ice-cold temperature and were further incubated with equal volumes of fluorescein isothiocyanate-bound mouse α Thy 1.2 antibody (1:280 dilution for T-cell staining; Beckton Dickinson Monoclonal Center Inc., Mountain View, CA) or the F(ab')₂ fragment of goat α mouse IgG [F(ab')₂] antibody (1:20 dilution for B-cell staining; Cappel Laboratories, Cochranville, PA) for 0.5 hr at ice-cold temperature. After washing, fluorescent cells were counted under a fluorescence microscope (Leitz Dialux 20 B: Ernst Leitz Wetzlar GMBH, Wetzlar, West Germany), and more than 200 cells were scored (Table 1). Administration of 0.25 ml of rabbit α mouse thymocyte globulin eliminated T-cells but not B-cells in the spleen. It also eliminated T-cells in the blood resulting in the decrease of T-cell. Although blood leukocyte recovery was variable among the experiments, there was not much difference in blood B-cell concentrations in each of the 3 experiments among mice administered rabbit α mouse thymocyte globulin or rabbit normal globulin, or left untreated. These results show that rabbit α mouse thymocyte globulin selectively eliminated T-cells without affecting B-cells in the spleen and blood of BALB/c mice.

IFN Concentration in the Serum. IFN concentrations of the serum were measured according to the previously reported procedure (7).

RESULTS

Therapeutic Effects of IFN in Meth A-bearing Mice. BALB/c mice inoculated i.d. with 2 x 10⁵ Meth A cells were administered 2 x 10⁶ units of IFN i.p. or i.v. for 10 days starting 1 day after Meth A inoculation. The antitumor effect in the mice was measured by the tumor size at different intervals and also by tumor weight at the termination of the experiment (Chart 1). IFN administered i.v. suppressed the tumor growth, whereas the suppressive effect of IFN administered i.p. was not statistically supported. The efficacy of IFN administered i.v. was reproducible, as will be summarized later in Table 3.

![Chart 1. Therapeutic effect of IFN in Meth A-bearing mice. BALB/c mice inoculated i.d. with 2 x 10⁵ Meth A cells were not treated (□), or administered 2 x 10⁶ units/mouse/day of IFN i.p. (△) or i.v. (■) for 10 consecutive days starting 1 day after tumor inoculation. Tumor sizes were calculated as indicated and, at the termination of the experiment, the weights of tumors excised from the mice were measured and expressed as the mean ± S.D. * statistical significance at p < 0.05 by the t test versus the control group (non-treated). S.D.s were < 0.05% (i.p. IFN group), and < 40% (i.v. IFN group) of the mean values.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor size (mean ± S.D., mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15.1 ± 1.7</td>
</tr>
<tr>
<td>IFN</td>
<td>10.2 ± 2.7</td>
</tr>
<tr>
<td>IFN, rabbit normal globulin</td>
<td>10.6 ± 1.7</td>
</tr>
<tr>
<td>IFN, rabbit α mouse thymocyte globulin</td>
<td>15.5 ± 0.6</td>
</tr>
</tbody>
</table>

* Measured at 16 days (Experiment 1), 14 days (Experiment 2), and 17 days (Experiment 3) after Meth A inoculation. Mean ± S.D. of 6 to 7 mice. Statistically significant at p < 0.05 by the t test versus "None" or "IFN, rabbit α mouse thymocyte globulin."
Table 3
Failure of IFN therapy in Meth A-bearing nude mice
Meth A-bearing BALB/c nude mice (nu/nu) and Meth A-bearing BALB/c mice (+/+), were not treated or were treated with IFN by the same regimen as that of Chart 2. The therapeutic effect was expressed by the tumor size at the indicated intervals in which the IFN-dependent therapeutic effect was maximal.

<table>
<thead>
<tr>
<th>Tumor size ((\bar{x}) ± S.D., mm) in:</th>
<th>IFN-treated mice</th>
<th>Non-treated mice</th>
<th>% of tumor suppression</th>
<th>Mean of 4 experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>nu/nu mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>18.4 ± 0.7 (^a)</td>
<td>20.5 ± 1.4</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>19.4 ± 1.3</td>
<td>20.7 ± 1.3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Experiment 3</td>
<td>19.9 ± 1.3</td>
<td>20.7 ± 1.7</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Experiment 4</td>
<td>21.9 ± 0.8 (^b)</td>
<td>24.3 ± 1.5</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>+/+ mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>9.9 ± 3.9 (^a)</td>
<td>15.4 ± 3.7</td>
<td>35.8</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>9.5 ± 4.2 (^a)</td>
<td>16.5 ± 2.1</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>Experiment 3</td>
<td>12.4 ± 3.2 (^a)</td>
<td>17.4 ± 1.7</td>
<td>29.8</td>
<td></td>
</tr>
<tr>
<td>Experiment 4</td>
<td>13.4 ± 6.8 (^a)</td>
<td>19.2 ± 2.0</td>
<td>30.3</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D. of 6 to 7 mice measured at 16 days (nu/nu mice, Experiment 1), 17 days (nu/nu mice, Experiments 2 and 3), 18 days (nu/nu mice, Experiment 4; +/+ mice, Experiments 1, 2, and 3), and 19 days (+/+ mice, Experiment 4).

\(^b\) Calculated according to:

\[
\frac{1}{\text{Mean tumor size of IFN-treated mice}} - \frac{1}{\text{Mean tumor size of non-treated mice}} \times 100
\]

Statistically significant at \(p < 0.05\) by the t-test versus nontreated mice.

Statistically significant at \(p < 0.05\) by the t-test between nu/nu and +/+ mice.

T-Cell Involvement in the IFN-dependent Therapeutic Effect.
Administration i.p. of rabbit \(\alpha\) mouse thymocyte globulin, but not rabbit normal globulin, completely nullified the i.v. IFN-dependent therapeutic effect (Table 2; Chart 2), 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 nude mice, athymic and defective in T-cell-mediated immunity.

Two strains of mice, T-cell-competent BALB/c (+/+), and BALB/c nude (nu/nu), bearing Meth A tumor, were administered IFN i.v. and examined for tumor size (Chart 3). IFN retarded the tumor growth in T-cell-competent BALB/c mice; in 3 of 6 of this group, the tumor was either stable for a prolonged time (2 mice) or regressed (1 mouse) in this particular experiment. In nude mice, IFN marginally retarded the tumor growth, and this was to a much lesser extent than that in the BALB/c (+/+). The summarized results of the 4 separate experiments are presented (Table 3). Although in 2 of 4 experiments, IFN was effective in Meth A-bearing nude mice, the suppression in these mice was marginal and was much lower than that achieved in +/+ mice. These results are consistent with the finding that rabbit \(\alpha\) mouse thymocyte globulin nullified the IFN-dependent therapeutic effect and suggest that host T-cells were responsible for the IFN-dependent therapeutic effect. These results suggest the small, if any, contribution of the antiproliferative activity of IFN to the therapeutic effect and support the hypothesis that T-cell-associated immunity was involved in the IFN-dependent therapeutic effect.

Lack of Correlation of in Vivo Therapeutic Effect with in Vitro Antiproliferative Activity of IFN in Different Tumors.
In the subsequent experiments, it was examined whether the antiproliferative activity of IFN directed to tumor cells was involved in the IFN-dependent therapeutic effect. To find a clue in this regard, the antitumor activity of IFN was examined in another 2 tumors in BALB/c mice (Table 4). Under the same (Protocol 2) or a similar regimen (Protocol 1) used in the Meth A experiment, IFN was not effective (Protocol 1) or was marginally effective, if at all, (Protocol 2) in Colon 26 adenocarcinoma, whereas it was more markedly effective in Meth 1 fibrosarcoma (using both protocols).

In vitro antiproliferative activities of IFN were examined in 3 tumors, and their IFN sensitivities were compared with the IFN-dependent therapeutic effect in mice bearing these tumors (Table 5). Colon 26 tumor was the most sensitive to IFN in the in vitro suppression of cell proliferation; however, it was the least responsive to IFN therapy. Meth 1 tumor was much more resistant to IFN than Meth A tumor (100-fold difference in 50% inhibition...
T-CELL-DEPENDENT THERAPY WITH IFN

Table 4
Antitumor activity of IFN in Colon 26- and Meth 1-bearing mice
Mice inoculated i.d. with 10⁶ Colon 26 and 10⁶ Meth 1 cells were administered 10⁵ units/mouse/day i.v. for 13 days (Protocol 1) or 2 x 10⁵ units/mouse/day for 10 days (Protocol 2) and examined for tumor size.

<table>
<thead>
<tr>
<th>Mice bearing:</th>
<th>Tumor size (diameter, mm) of tumor bearings treated with:</th>
<th>% of control tumor size of IFN-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 1</td>
<td>Nothing</td>
<td>MN</td>
</tr>
<tr>
<td>Colon 26</td>
<td>11.5 ± 0.6a</td>
<td>11.0 ± 0.7</td>
</tr>
<tr>
<td>Meth 1</td>
<td>13.5 ± 1.3</td>
<td>10.8 ± 1.4</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>Colon 26</td>
<td>15.3 ± 0.8</td>
</tr>
<tr>
<td>Meth 1</td>
<td>16.3 ± 1.1</td>
<td>12.4 ± 2.9</td>
</tr>
</tbody>
</table>

* Significant suppression at p < 0.05 by the t test.

The results of Table 2 and Charts 1 to 3 are arbitrarily expressed. "++" indicates a stronger tumor suppression than "+.

Table 5
In vitro antiproliferative activity of IFN in 3 murine tumor cells
Tumor cells (2 x 10⁵/1ml in Meth A and Meth 1; 1 x 10⁵/1ml in Colon 26) were incubated in vitro with different concentrations of IFN for 4 days. The cell concentrations were measured on a Coulter Counter.

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>IFN concentration required for inhibiting cell proliferation by 50% of control (units/ml)</th>
<th>Reference:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon 26 adenocarcinoma</td>
<td>176 ± 72²</td>
<td>Reference:</td>
</tr>
<tr>
<td>Meth A fibrosarcoma</td>
<td>518 ± 165²</td>
<td>Reference:</td>
</tr>
<tr>
<td>Meth 1 fibrosarcoma</td>
<td>50,750 ± 14,899²</td>
<td>Reference:</td>
</tr>
</tbody>
</table>

IFN Concentration in the Serum. The contribution of the antiproliferative activity of IFN to its therapeutic effect in tumor-bearing mice was further tested by measuring IFN concentrations in the serum (Table 6). IFN was injected i.v. into Meth A- and Colon 26-bearing mice; and at 1-, 3-, and 6-hr intervals, IFN concentrations were measured. In Meth A-bearing mice, IFN was detected in the serum at all 3 intervals, but IFN concentrations at 3- and 6-hr intervals were lower than the 50% inhibition concentration of Meth A cells. After 10 injections of IFN, serum IFN concentration was not elevated at 3- and 6-hr intervals. These low concentrations and the fast clearance of IFN may not guarantee the involvement of IFN-dependent antiproliferative activity in its therapeutic effect. Furthermore, no difference was found between Colon 26- and Meth A-bearing mice in IFN concentrations of the serum. Since Colon 26 cells were more sensitive to the antiproliferative activity of IFN than Meth A cells (Table 5), IFN should have been therapeutically more effective on Colon 26 tumor than on Meth A tumor, if the antiproliferative activity of IFN was mainly responsible for its therapeutic effect.

DISCUSSION

The present study showed that mouse IFN was therapeutically effective in mice inoculated i.d. with solid tumors. The in vivo antitumor activity of IFN was analyzed from the viewpoint of antiproliferative activity and host-mediated activity. Our hypothesis that the IFN-dependent therapeutic effect is host T-cell immunity-mediated was based on the following 2 findings. Rabbit α mouse thymocyte globulin completely nullified the IFN-dependent therapeutic effect. In nude mice, the therapeutic effect of IFN was marginal, if present at all, and much less effective than in T-cell-competent (+/+) mice.

An earlier study reported that rabbit α mouse thymocyte globulin did not nullify the IFN-dependent therapeutic effect in mice bearing L1210 leukemia and Ehrlich cells (5). This is in sharp contrast with the present results. In the Meth A tumor, antitumor effector immunocytes involved in the tumor rejection were identified as T-cells (11), and this is consistent with the present finding, i.e., successful abrogation of the IFN-dependent therapeutic effect by α mouse thymocyte globulin. In L1210 leukemia and Ehrlich tumor, the antitumor effector immunocytes may not be T-cells. Although we reported previously that the antitumor effector cells in mice hyperimmunized with 10⁷ live L1210 cells were T-cells (8), the L1210 cells used by earlier investigators seem to be different from the ones currently supplied to us by the National Cancer Institute, as indicated by their prolonged survival days. Mice inoculated with 10⁷ L1210 cells lived for about 24 days in the earlier study (12), whereas it was about 10 to 12 days with our currently used L1210 cells. It was clearly demonstrated that, under the different experimental conditions including the use of L1210 subclones, the tumor was rejected by a host immunological system other than one involving T-cells (3, 10, 12). Thus, we need further information on the

Table 6
IFN serum concentration in mice after a single or 10 administrations of IFN
Mice were inoculated i.d. with 2 x 10⁵ Meth A cells and 1 x 10⁶ Colon 26 cells. Ten days later, these mice were administered 2 x 10⁵ units/0.2 ml/mouse of IFN i.v. Alternatively, Meth A-bearing mice were administered 2 x 10⁵ units/0.2 ml/day/mouse of IFN i.v. for 10 consecutive days starting 1 day after Meth A inoculation; at different intervals after the last IFN administration, their sera were harvested and measured for IFN concentrations. Each point shows the summarized data from 3 mice in a single-shot experiment or 5 mice in a 10-shot experiment.

<table>
<thead>
<tr>
<th>IFN serum concentration of the following intervals after IFN administration at:</th>
<th>Day 10</th>
<th>Days 1 to 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>3 hr</td>
</tr>
<tr>
<td>Meth A</td>
<td>1938 ± 370²</td>
<td>323 ± 19²</td>
</tr>
<tr>
<td>Colon 26</td>
<td>1943 ± 328²</td>
<td>219 ± 19²</td>
</tr>
</tbody>
</table>

* Mean ± S.D. of 3 mice.  
² Mean ± S.D. of 5 mice.
antitumor immunocyte populations before drawing any conclusion on the basis of the effect of immunological agents.

A recent study showed that IFN induced tumor necrosis in which host immunocyte infiltration was not involved and proposed that IFN-dependent elimination of solid tumors was due to mechanisms that were not seriously considered heretofore (1). However, it was demonstrated in tumor necrosis that tumor regression was triggered by T-cell-dependent immune response involving the recognition of tumor-associated antigen (4). Thus, further experimentation on T-cell immunity will be worth undertaking before testing for alternative antitumor mechanisms in the IFN-dependent therapeutic effect.

The present study demonstrated the involvement of T-cell immunity in the IFN-dependent therapeutic effect. However, it did not identify IFN-triggered immunological phenomena. IFN may modify tumor cells and/or host immunity. A recent study showed that Meth A cells incubated in vitro with IFN lost malignancy and that IFN caused a reduction in the expression of the tumor-specific transplantation antigen, but increased the expression of H-2 antigens, suggesting that the increased expression of H-2 antigens could lead to an increased frequency of recognition by T-cells (14). This may be involved in the IFN-dependent therapeutic effect in the present study.

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