Enhanced in Vivo Therapeutic Response to Interferon in Mice with an in Vitro Interferon-resistant B-Cell Lymphoma

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ABSTRACT

A stable subline of 38C13 B-cell lymphoma (SIR-1) resistant to the antiproliferative effects of α-interferon (IFN) was isolated. In addition to defects in antiproliferative effects of IFN, SIR-1 is defective in IFN-mediated antiviral activity against both encephalomyocarditis virus and vesicular stomatitis virus. It is also defective in the induction of 2'-5'-oligoadenylate synthetase mRNA and enzyme activity, enhancement of H-2 antigen expression, and transient induction and subsequent repression of c-myc by IFN. SIR-1, although completely resistant to IFN in vitro, is more sensitive to IFN than the parental cell line in vivo. IFN treatment at 10^5 units, three times weekly, resulted in a 28% increase in mean survival time and a 1.4% long term survival rate in the IFN-sensitive 38C13 cell line but resulted in a 275% increase in mean survival rate and a 27% long term survival rate in the interferon-resistant SIR-1 mutant. Statistical analysis of 38C13 and SIR-1 with and without IFN treatment demonstrate that: a) the SIR-1 mutant remains sensitive to the cytotoxic effects of IFN in vivo (P < 0.0001); and b) the mean survival and long term survival of animals with the SIR-1 mutant is significantly greater than for animals with the IFN-sensitive 38C13 cell line (P < 0.0001). Two additional independently isolated IFN-resistant cell lines (SIR-111 and SIR-E102) also demonstrate significantly enhanced in vivo response to IFN compared to the interferon-sensitive parental (38C13) cells. These results indicate that, for this cell line, the antitumor effects of IFN are mediated by activation of host defenses and that resistance to the in vitro cytotoxic effects of IFN results in a tumor phenotype that is more readily recognized by host defenses and eliminated.

INTRODUCTION

IFN is currently the treatment of choice for hairy cell leukemia, and various other tumors are sensitive to the therapeutic effects of interferon (1). Resistance to IFN can arise in tumor cell lines in culture and in IFN-treated patients, limiting its usefulness as a therapeutic agent (1–3). Since the experimental evidence suggests that both direct cellular and host-mediated mechanisms may be involved in the interferon response induced by IFN, resistance could arise either in the direct cytotoxic pathways or to the host defense mechanisms (4–6). It would be useful, therefore, to understand how specific genes regulated by IFN relate to the direct antiproliferative and host-mediated antitumor response mechanisms and to determine how the expression of these genes is altered in cells which have become resistant to IFN.

Interferons can alter numerous cellular processes including establishment of antiviral, antiproliferative, and antitumor activity, and modulation of immune function and cell differentiation (7, 8). A number of interferon-regulated genes have been identified and cloned including 2'-5'-oligoadenylate synthetase, c-myc, histocompatibility antigens, and a variety of cDNAs which code for proteins of unknown function (1, 7, 9). The function of some of these proteins is at least partially understood. The role of 2'-5'-Oligoadenylate synthetase may help establish an antiviral state against some viruses (7). c-myc is a nuclear protooncogene with transforming capabilities, especially when overexpressed in the presence of other oncogenes, such as H-ras, and the histocompatibility antigens are involved in immune recognition. However, the importance of IFN control of these and other proteins in regulation-specific cellular processes mediated by IFN remains elusive, especially with regard to the antiproliferative and antitumor effects of IFN.

While the antiproliferative effects of interferon in vitro are incompletely understood, the antiproliferative and antitumor activity of interferon in vivo may have additional levels of complexity. Several reviews have summarized the evidence that interferon inhibits the growth of virus-induced and spontaneous tumors in animals and the data suggest that, depending on the system, interferon exerts both direct antitumor effects and indirect, host-mediated defenses (4–6, 10). The host-mediated defenses may include enhanced macrophage and killer cell antibody-dependent cellular cytotoxicity, increased NK cell function, and enhanced major histocompatibility and Fcγ receptor expression (1, 5, 8); however, the distinction between the host-mediated and direct antitumor effects of IFN may be blurred since IFN may act directly on the cell in ways that change its response to host defenses. For example, while induction of H-2 antigens by IFN may not be important in the direct antitumor effects of IFN, it may still be important in the interaction of the tumor cells with host defense mechanisms. Thus, resistance to the cytotoxic effects of IFN may result from: a) loss of direct cytotoxic effects of IFN; b) altered recognition and lysis by host defenses; or c) altered interaction between the IFN-stimulated tumor cell and the IFN-stimulated host. It would be useful, therefore, to study the problem by dissecting these possible pathways genetically.

To analyze the role of specific interferon-regulated genes on direct antitumor and host-mediated antiproliferative and antitumor mechanisms, we have used a model system based on a B-cell tumor that is sensitive to the effects of interferon both in vitro and in vivo. Variant cell lines resistant to the in vitro cytotoxic effects of IFN were isolated, and individual clones were analyzed for the induction of antiviral and antiproliferative states by IFN and for the expression of the mRNA which encode 2'-5'-oligoadenylate synthetase, c-myc, and H-2 antigens. Finally, three independently derived IFN-resistant clones were injected into animals to determine the in vivo therapeutic response to IFN.

MATERIALS AND METHODS

Cell Culture. A carcinoma [dimethylbenz(a)anthracene]-induced B-cell lymphoma, 38C13, was produced in a C3H/eB mouse depleted of
T-cells (11, 12). This tumor and its in vivo-adapted cell line express IgM with a unique idiotype. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, glutamine, amino acids, and β-mercaptoethanol, as described previously (5). Cells were screened for Mycoplasma contamination and were found to be negative. Cell counts were made on a hemocytometer using the trypan blue dye exclusion technique. Clones were selected in soft agarose system as described previously (4), containing IFN at 10,000 units/ml. Briefly, a bottom layer of agarose (1.0%) was prepared in complete medium and allowed to harden before pouring a top layer of 0.4% which contained the cells pretreated for 2 h with IFN. Cells were mutagenized by treating them with 0 to 350 ng/ml of EMS for 6 h. The cells were then washed and then thawed to lyse the cells, and the EMC virus yield was measured by titration of viral hemagglutinin. Virus from control and IFN-treated cells were diluted by serial 2-fold dilution in round-bottomed 96-well microtiter plates. Human type O erythrocytes were added and the plates were incubated at room temperature for 2 h. The dilution at which hemagglutination occurs is recorded as the titer for the virus. VSV was titrated by incubation of 3 × 10⁶ cells in the presence or absence of 1 × 10⁹ units/ml of interferon for 18 h. The cells were washed and counted and 5 × 10⁶ cells were incubated with 0.5 ml of a crude VSV preparation for 45 min. The cells were washed and incubated in complete medium for various lengths of time and then frozen at −80°C. Dilutions of virus were titrated on mouse L-cells and plaques were counted after 48 h.

Interferon. Pure preparations of human recombinant hybrid IFN-αA/D (Hoffmann-LaRoche Inc., Nutley, NJ; specific activity, 7 × 10⁶ units/mg) was supplied by the American Cancer Society.

In Vivo Studies. C3H/HeN mice were purchased from Simonsen Laboratory, Gilroy, CA. Mice were between 7 and 9 weeks of age when used in therapy experiments. Groups of 10 mice were given injections of 5 × 10⁶ tumor cells i.p. and therapy was begun 48 h later. Preliminary experiments were performed evaluating 10⁶–10⁷ units/mouse of IFN against the SIR-1 cells in vivo, and the results indicated that a 10⁴-unit/mouse dose is optimal. This is consistent with our previously published results comparing the effects of a range of doses of all 3 types of IFN on 38C13 cells (15). IFN (10⁴ units/mouse) was injected i.p. and continued as a three times weekly schedule for 3 weeks. Control animals were treated with vehicle only. Mice were monitored for time of survival.

Fluorescein Labeling and FACS Analysis. Aliquots of 5 × 10⁶ cells were stained for 30 min on ice with monoclonal IgG2a anti-idiotype S1C5 (1 μg), 111.1 IgG2a anti-H-2Kb (1 μg), or control IgG2a monoclonal antibody (1 μg). The cells were washed twice in RPMI 1640 (Gibco) with 10% fetal calf serum. Fluorescein isothiocyanate-labeled goat anti-mouse γ chain-specific IgG (Southern Biotechnology Associates, Birmingham, AL) was added (15 μl of appropriate dilution) for an additional 30 min on ice. The cells were then washed twice and fixed with 1% paraformaldehyde in phosphate-buffered saline. The fluorescence per cell was determined with a fluorescence-activated cell sorter (FACS 440; Becton-Dickinson), and a histogram showing the number of stained cells against the intensity of fluorescence was recorded.

RNA Isolation and Analysis. RNA was prepared by the guanidium-ethanol precipitation method described by Chirgwin et al. (16), except that the RNA pellet was digested with proteinase K and reprecipitated in guanidium HCl prior to the final salt precipitation step to remove contaminating proteins. RNA was glyoxylated, electrophoresed, transferred to Nytran paper, and washed as recommended by the manufacturer. The 2'-5'-oligoadenylate synthetase probe, which recognizes 1.7- and 4.0-kilobase mRNA species in mouse cells, was provided by Dr. Bryan Williams. The ferritin heavy chain probe, which recognizes a 1.0-kilobase mRNA species in mouse cells, was provided by Dr. Frank Torti, and the c-myc probe, which recognizes a 2.4-kilobase mRNA species in mouse cells, was obtained from Amersham. The probes were labeled by random priming, using the Boehringer Mannheim random prime labeling kit, and purified by spin-column centrifugation according to the method of Maniatis et al. (17).

Statistical Analysis. Survival times for 38C13 and SIR-1 cell lines were analyzed by computing the means ± SE for the control and IFN-treated populations. Two-sample t tests and Wilcoxon signed-rank tests were used to compare the populations. The Wilcoxon signed-rank test is particularly useful in cases where individual values may greatly exceed the mean value of the population, resulting in a skewed distribution curve. This occurs in our case due to the long term survivors in the IFN-treated groups and can be compensated for by using a ranking test. Both t tests and Wilcoxon signed-rank tests were performed on all samples and yield essentially equivalent results. Long term survivors are defined as those animals living greater than 90 days, and for the purpose of statistical analysis for the t tests, these animals are assigned a value of 90 days. Extensive previous experience has demonstrated that animals that survive longer than 90 days have essentially a normal life span (5, 19). Long term survival rates of 38C13 and SIR-1 cells were compared using a χ² test. The analysis was performed using the StatView statistical analysis program.

RESULTS

In Vitro Cytostatic and Cytotoxic Effects of Interferon against 38C13 Cells. Human hybrid recombinant IFN was tested for direct antiproliferative activity against 38C13 cells over the range of 0–10,000 units/ml. The results, shown in Fig. 1, indicate that as little as 0.1 unit/ml of IFN will reduce the total accumulation of cells by 50% over 4 days of continuous treatment.

The time required for interferon to induce cytotoxic action was determined by treatment of 38C13 cells with 1000 units/ml of IFN. Aliquots of cells were removed daily and counted. These results, shown in Fig. 2, indicate that cytostatic activity is evident by 24 h and that cytotoxic activity is evident by 48 h. No detectable surviving cells were visible after 4 days of continuous exposure to IFN.

Isolation of Interferon-resistant Cells. To determine the frequency of surviving cells following continuous exposure to high doses of IFN and to isolate IFN-resistant clones, 38C13 cells were treated with 10,000 units/ml of IFN and plated in dual layer soft agarose (Table 1). The frequency of resistance to IFN was determined by dividing the number of surviving colonies by the number of starting cells. The number of starting cells...
were found to remain resistant to 100,000 units/ml of IFN for 10 units/ml IFN, and plated in soft agarose.

Cells were observed in the IFN-treated cultures from these cells were treated with or without IFN (10,000 units/ml). The cells were counted 3 days later. From 4 to 6 x 10^5 cells/ml were treated daily with 1000 units/ml of interferon. Aliquots of cells were removed at daily intervals and counted. Data are presented as a percentage of the initial number of cells.

were resistant to infection with EMC even without IFN treatment. The six mutant cell lines were readily infected by EMC viruses. The six mutant cell lines were readily infected by EMC virus (multiplicity of infection, 10:1). Virus titers were determined by plaque formation on mouse L-cells. PFU, plaque-forming units.

SIR-1 and SIR-2, were further analyzed for IFN-induced resistance to VSV infection. These results, shown in Table 2, again indicate that the parental cell line does not support virus replication, even in the absence of IFN treatment. Both variants, however, readily support virus replication and IFN treatment is incapable of inducing an antiviral state in these cells.

Table 2 Characterization of Stanford IFN-resistant (SIR) clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IFN</th>
<th>2′-5′A* (pmol/h/10^5 cells)</th>
<th>VSV titer</th>
<th>PFU/ml</th>
<th>FACSProfile</th>
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<tbody>
<tr>
<td>38C13</td>
<td>-</td>
<td>8.4</td>
<td>0</td>
<td>3.0</td>
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<td>22.8</td>
<td>0</td>
<td>3.0</td>
<td>87.9</td>
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<tr>
<td>SIR-1</td>
<td>-</td>
<td>0.3</td>
<td>1:16</td>
<td>4.7</td>
<td>93.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.8</td>
<td>1:16</td>
<td>4.7</td>
<td>92.8</td>
</tr>
<tr>
<td>SIR-2</td>
<td>-</td>
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<td>1:16</td>
<td>5.2</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>1:16</td>
<td>5.5</td>
<td>86.9</td>
</tr>
<tr>
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<td>1:16</td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>1:16</td>
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<tr>
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<td>0.3</td>
<td>1:8</td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.3</td>
<td>1:8</td>
<td></td>
<td>NT</td>
</tr>
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<td>1.7</td>
<td>1:16</td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td></td>
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<td>1:16</td>
<td></td>
<td>NT</td>
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<td>SIR-18</td>
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<td>1:8</td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.6</td>
<td>1:8</td>
<td></td>
<td>NT</td>
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</table>

* Cell extracts with and without IFN treatment (10^3 units/ml for 24 h) were analyzed for 2′-5′A activity (pmol/h/10^5 cells) using [3H]ATP as substrate. Values presented are the means of three independent experiments performed in triplicate.

Two IFN-resistant mutants, SIR-1 and SIR-2, were further analyzed for IFN-induced resistance to VSV infection. These results, shown in Table 2, again indicate that the parental cell line does not support virus replication, even in the absence of IFN treatment. Both variants, however, readily support virus replication and IFN treatment is incapable of inducing an antiviral state in these cells.

Induction of 2′-5′-Oligoadenylate Synthetase in the Parental and Mutant Cell Lines. 2′-5′-Oligoadenylate synthetase is an enzyme that is induced by IFN and will polymerize ATP into 2′-5′A when activated by double stranded RNA. 2′-5′-Oligoadenylate synthetase activity was measured in the parental cell line and 7 variant cell lines. The results, shown in Table 2, indicate that the parental cell line contains 8 pmol/h/10^5 cells in the absence of IFN treatment and that this increases (288%) to 23 pmol/h/10^5 cells after 18 h of treatment with 100 units/ml of IFN. In contrast, no mutant cell line had measurable endogenous 2′-5′-oligoadenylate synthetase activity nor is there any induction of 2′-5′-oligoadenylate synthetase upon treatment with IFN. These results indicate not only that the variant cell lines have lost the capacity to induce 2′-5′-oligoadenylate synthetase but also that they have lost the endogenous levels.

To further characterize the induction of 2′-5′-oligoadenylate synthetase following IFN treatment, total mRNA preparations were made in the parental and SIR-1 cells at various times following IFN treatment. The results, shown in Fig. 3, indicate that 2′-5′-oligoadenylate synthetase mRNA is present in the parental cell line prior to IFN treatment. Both forms of the 2′-5′A mRNA are induced with 1 h of treatment with 100 units/ml of IFN. The larger mRNA species is more strongly induced in 1 h, while the smaller form is relatively more abundant in the 24-h treatment. In contrast, the mutant cell line, SIR-1, has no detectable endogenous expression of either form of the 2′-
enhanced response to interferon in B-cell lymphoma

In Vivo Effects of Interferon against 38C13 and SIR-1. IFN injected i.p. (10⁴ units/mouse, 3 times weekly for 3 weeks) can extend the mean survival time about 20% and cure about 5% of the mice which have the 38C13 tumor (5). To determine whether the basis of the therapeutic effect was mediated by direct cytotoxic effects of IFN against these cells, animals were given injections of 5 x 10⁴ 38C13 or SIR-1 cells and tested for their response to IFN. The composite of 7 experiments (approximately 10 animals/group) are shown in Table 3. IFN at 10⁴ units/mouse, 3 times weekly for 3 weeks, will increase the mean survival time of 38C13 treated animals from 21.5 ± 4.4 days to 27.8 ± 9.4 days, a 28% increase. There were no long term survivors (>3 months) in the untreated group and only 1 (1 of 68) long term survivor in the IFN-treated group. These results are comparable to previous results in this laboratory where 10⁴ units of IFN increased the mean survival time 20% and yielded about 3% long term survivors (5). Curiously, the SIR-1 variant, while resistant to IFN in vitro, is more responsive to IFN in vivo. The mean survival rate for the untreated animals was 18.1 ± 1.5 days, slightly shorter than for the 38C13 cell line, while that for the IFN-treated animals was 49.8 ± 25.2 days, a 275% increase. These results indicate that IFN increased the mean survival of animals with the 38C13 cell line by 6.3 days compared to a net increase of 31.7 days for animals with the SIR-1 cell line. To determine whether the increase in mean survival time observed in the population of animals given injections of the SIR-1 mutant was due to random chance, a t test was performed. The probability that a difference of this magnitude would occur as a result of random chance, with this number of animals, can be excluded to a level of less than 0.0001.

To further analyze the in vivo response to IFN for these two cell lines, the long term survival was compared. There were no long term survivors in the untreated group for animals given injections of either 38C13 or SIR-1 cell lines; however, the IFN-treated 38C13 group had a long term survivor frequency of 1.4% (1 of 68), and the IFN-treated SIR-1 animals had a long term survivor frequency of 26% (15 of 57). To determine whether the difference in survival frequency for IFN-treated animals with either the 38C13 or SIR-1 cell lines could be attributed to random chance, a X² test was performed. The results of this test indicate that the probability that this result arose as the result of random chance is less than 0.0001. These results further verify that the SIR-1 mutant remains sensitive to the cytotoxic effects of IFN in vivo and that the IFN-resistant SIR-1 mutant is more sensitive to the therapeutic effects of IFN in vivo than the IFN-sensitive 38C13 parental cell line.

In Vivo Effects of Interferon against SIR-111 and SIR-E102. The enhanced in vivo response to IFN could have been due to fortuitous isolation of a clone sensitive to the in vivo effects of IFN due to cellular changes unrelated to IFN resistance. To assess this possibility, two additional independently derived clones were isolated by placing 50 38C13 cells/plate (10 plates) or 50 EMS mutagenized 38C13 cells/plate (10 plates) and allowing the cells to grow to 5 x 10⁵ cells/plate before adding

Table 3 Comparison of survival of 38C13 and SIR-1 in vivo

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean survival days</th>
<th>% of long term survivors</th>
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</thead>
<tbody>
<tr>
<td>38C13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>68</td>
<td>21.5 ± 4.4</td>
<td>0</td>
</tr>
<tr>
<td>IFN</td>
<td>68</td>
<td>27.8 ± 9.4</td>
<td>1.4 (1/68)</td>
</tr>
<tr>
<td>SIR-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>57</td>
<td>18.1 ± 1.5</td>
<td>0</td>
</tr>
<tr>
<td>IFN</td>
<td>57</td>
<td>49.8 ± 25.2</td>
<td>27 (15/57)</td>
</tr>
<tr>
<td>SIR-111</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>16.9 ± 1.7</td>
<td>0</td>
</tr>
<tr>
<td>IFN</td>
<td>10</td>
<td>42.1 ± 33.4</td>
<td>30 (3/10)</td>
</tr>
<tr>
<td>SIR-E102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>22.1 ± 23.9</td>
<td>10 (1/10)</td>
</tr>
<tr>
<td>IFN</td>
<td>10</td>
<td>44.1 ± 32.1</td>
<td>30 (3/10)</td>
</tr>
</tbody>
</table>

* Mean survival time is the average number of days of survival for the specified groups of animals. Long term survival is determined as greater than 90 days, and for statistical analysis these animals are assigned a value of 90 days, although these animals live essentially a normal life span.

IFN (1 x 10⁴ units/mouse).

Fig. 3. Northern analysis of interferon-responsive genes. RNA from M. L. (mouse L929), 38C13, and SIR-1 was prepared at the times indicated and probed for (A) 2'-5' oligoadenylate synthetase (2-5A), c-myc, and ferritin and (B) H-2 mRNA expression.

5'-oligoadenylate synthetase mRNA and no detectable induction with IFN.

c-myc. Total mRNA was prepared from control and IFN-treated cells and analyzed for c-myc expression by Northern analysis. The results, shown in Fig. 3, indicate that c-myc is transiently induced 87% in the first h of treatment with 100 units/ml of IFN. This induction is followed by a repression of c-myc to 10% of control values by 24 h. These values were determined by scanning densitometry and normalized to ferritin mRNA expression, which is not altered by IFN treatment in these cells. The SIR-1 cell line, while having normal levels of c-myc expression, fails in repression of c-myc m-RNA expression following IFN treatment. There was no change in ferritin mRNA expression in the variant cell line compared to the parent.

Analysis of H-2 Antigens and Idiotype. Flow cytometry was used to evaluate the cell surface expressions of H-2 antigens and idiotype on the parental and variant cell lines. These results, shown in Table 2, indicate that while the surface presentation of idiotype and H-2 antigens is normal for these cells, the SIR-1 and SIR-2 cell lines have lost IFN-inducible enhancement of H-2Kβ and H-2Dα. The Northern blot shown in Fig. 3A was stripped and reprobed for H-2 mRNA expression, and the results, shown in Fig. 3B, indicate that there is no significant induction of H-2 mRNA in the 38C13 or SIR-1 cell lines following IFN treatment. Moreover, there is no appreciable difference in the level of expression of H-2 mRNA between the 38C13 and the SIR-1 cell lines.

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ENHANCED RESPONSE TO INTERFERON IN B-CELL LYMPHOMA

the selective media containing IFN. Single colonies were isolated from separate plates to assure that the IFN-resistant mutants arose independently. These mutants were also found to remain resistant to 10^6 units/ml of IFN for more than 30 passages in the absence of IFN. SIR-111 was isolated by IFN selection of the original 38C13 population. The SIR-E102 cell line was isolated from an EMS-mutagenized population of 38C13 cells. EMS is a chemical agent that causes point mutations in DNA and mutagenesis by this agent increases the frequency at which IFN resistance occurs in the 38C13 cell line by about 50-fold, indicating that point mutations in DNA are sufficient to induce resistance to the cytotoxic effects of IFN. SIR-111 and SIR-E102 were injected into animals and treated with IFN as in the previous experiment. The results (Table 3) indicate that the mean survival time in the SIR-111 cell line is increased by 249% (from 16.9 ± 1.7 days to 42.1 ± 33.4 days) by treatment with IFN. The possibility that IFN is not increasing mean survival time can be rejected at the 99% confidence level using a Wilcoxon signed-rank test (P < 0.005). There were no long term survivors in the SIR-1 cell line without IFN treatment (0 of 10) while IFN treatment increased long term survival to 30% (3 of 10). In addition, IFN significantly increased the mean survival of animals with the SIR-E102 cell line from 22.1 ± 23.9 days to 44.1 ± 32.1 days, a 224% increase (P < 0.01).

Fewer animals (10/group) were analyzed for the SIR-111 and SIR-E102 cell lines than for the 38C13 and SIR-1 cell lines (60–70/group). There is some variation in the mean and long term survival times of the SIR-111 and SIR-E102 mutants compared to the SIR-1 mutant. This variation may simply reflect statistical variation due to the smaller sample groups for SIR-111 and SIR-E102; however, within each group IFN significantly increases the mean survival of the animals (Fig. 4).

The long term survival rate for animals with the SIR-E102 cell line was 20% (2 of 10) in the IFN-treated group compared to 10% (1 of 10) in the untreated group. The long term survival of the IFN-treated SIR-111 and SIR-E102 (6 of 20) can be compared to the long term survival of the IFN-treated 38C13 (1 of 68) cell line by χ^2 analysis. The results demonstrate that, like SIR-1, the independently derived IFN-resistant SIR-111 and SIR-E102 are significantly more sensitive to the effects of IFN in vivo than the IFN-sensitive 38C13 cell line (P < 0.005). One animal in the SIR-E102 group was a long term survivor despite the fact that the rest of the animals in that group died at 14.6 ± 1.2 days. We do not know why this one animal survived; it may simply have not received an injection of tumor cells. Regardless of the reason for the survival of this single animal, the increase in mean survival and the increase in the long term survival remain statistically significant for these two groups.

Pathological Analysis. Tissue samples were prepared for pathological analysis from the spleen, lung, liver, thymus, and lymph nodes of control and IFN-treated C3H mice given injections of 38C13 or SIR-1 cells. From 3 to 5 animals were analyzed in each group. While there was no evidence of tumor infiltration in the spleen or the lungs for either the 38C13 cells or the SIR-1 cells the liver, thymus, and lymph nodes were characterized as infiltrated to replace for both the 38C13 and SIR-1 cells in the untreated animals. In contrast, the liver, thymus, and spleen of IFN-treated animals were characterized as normal, verifying pathologically the antitumor effects of IFN against these cell lines. In addition, we have observed that the SIR-1 cell line grows as an ascites in C3H/HeN mice not treated with IFN, while the parental cell line forms solid nodules. In IFN-treated C3H/HeN mice, SIR-1 cells form solid nodules in the peritoneum. The altered pathological presentation of the SIR-1 mutant appears to be another manifestation of IFN resistance in these cells since the SIR-111 and SIR-E102 cell lines show equivalent growth patterns. This suggests that the nature of the IFN response in the host may have additional levels of complexity. Despite the fact that these tumors grow differently in vivo, the SIR-1 cell line is at least as aggressive as the 38C13 cell line in killing these animals if no IFN is given. Yet, IFN treatment is more effective against the IFN-resistant SIR cell lines than against the IFN-sensitive 38C13 cell line.

DISCUSSION

The SIR clones (1, 2, 7, 12, 16, and 18) were selected for resistance to the cytostatic and cytotoxic effects of IFN and are resistant to 100,000 units/ml of IFN in vitro, while the parental (38C13) cell line is inhibited in growth (>50%) by 0.1 unit/ml. Further analysis demonstrated that these IFN-resistant cell lines had lost not only the capacity to develop an IFN-induced antiviral state to VSV and EMC viruses but also the natural antiviral state of the parental cell line. Consistent with the loss of the antiviral state was the finding that each of the resistant clones had lost the high endogenous levels of 2′-5′-oligoadenylate synthetase present in the parent as well as the capacity to induce synthesis of the enzyme with IFN. The high levels of 2′-5′-oligoadenylate synthetase enzyme activity and mRNA expression in the parental cells, even without IFN treatment, suggest that these cells may be producing endogenous IFN. Various investigators have reported the production of endogenous IFN (1, 17) and have suggested that this may be important in the regulation of cell proliferation, differentiation, and the antitumor response, perhaps through its regulation of 2′-5′-oligoadenylate synthetase (7, 18). The results presented here demonstrate that in vitro expression of 2′-5′-oligoadenylate synthetase in response to IFN is not required for the antitumor response of IFN; moreover, in vitro responsiveness to IFN, from either endogenous or exogenous sources, is not critical for the antitumor effect of IFN against the SIR-1 cells.

Previous work in this and other laboratories has demonstrated that IFN can be an effective therapeutic agent for some
ENHANCED RESPONSE TO INTERFERON IN B-CELL LYMPHOMA

cancers (1, 2). In particular, we have recently demonstrated that IFN, used in conjunction with antidiotype antibodies, can result in an 80–90% long term survival rate in mice carrying the 38C13 B-cell lymphoma, while neither IFN nor the antidiotype antibody alone resulted in a more than 3–10% rate of long-term survivors (6, 19). Since IFN alone was cytotoxic to 38C13 cells in vitro, it was possible that direct cytotoxic effects of IFN on these cells were responsible for the therapeutic effects of IFN either as a specific agent or in conjunction with the antidiotype antibody; however, the results presented here argue that the direct anticalcular effects of IFN play little role in the in vivo therapeutic response. IFN treatment at 10⁴ units, three times weekly, resulted in a 28% increase in mean survival time and a 1.4% long term survival rate in the IFN-sensitive 38C13 cell line but resulted in a 275% increase in mean survival rate and a 25% long term survival rate in the interferon-resistant SIR-1 mutant. Seven independent experiments totaling 60–70 mice for both 38C13 and SIR-1 with and without IFN treatment have been evaluated and the results clearly demonstrate that: a) the SIR-1 mutant remains sensitive to the cytotoxic effects of IFN in vivo (P < 0.0001); and b) the mean survival and long term survival of animals with the SIR-1 mutant are significantly greater than those for animals with the IFN-sensitive 38C13 cell line. Mean survival was analyzed using a t test and Wilcoxon signed-rank test. Both tests yield the same result (P < 0.0001). The long term survival was analyzed using a χ² test (P < 0.001). These results provide compelling statistical evidence that the IFN-resistant SIR-1 mutant not only remains sensitive to the therapeutic effects of IFN in vivo but is significantly more responsive to the in vivo therapeutic effects of IFN at this dose than the IFN-sensitive 38C13 cell line.

The observed increase in in vivo sensitivity to 10⁴ units/mouse of IFN of the SIR-1 mutant is not due to a decrease in metastatic potential or aggressiveness of the cell line since the mean survival time of mice given injections of the parental (38C13) cell line without IFN was 21.6 ± 4.4 days, while the mean survival time for the mice given injections of SIR-1 was even shorter, 18.1 ± 1.45 days. This change, while only 3.5 days, is statistically significant (P < 0.0001), indicating that the SIR-1 cell line is more aggressive than the 38C13 cell line. There were no survivors in either the 38C13 or SIR-1 groups in the absence of IFN treatment. Moreover, the SIR-1 cell line is equally as sensitive if not more sensitive to the combination of IFN and antidiotype antibody, than the parental (38C13) cell line (19). The enhanced in vivo response to IFN is not due to an artifact of the individual cell clone since two additional, independently isolated IFN-resistant cell lines (SIR-111 and SIR-E102) also demonstrate significantly enhanced in vivo response to 10⁴ units/mouse of IFN compared to the parental, IFN-sensitive cell line.

Other investigators have also found that cells resistant to IFN in vitro remain sensitive to IFN in vivo. Belardelli et al. (10, 20) have reported the isolation and analysis of an interferon-resistant cell line derived from IFN-sensitive Friend leukemia cells. They showed that IFN could inhibit the growth of the primary tumor and its metastases of both IFN-sensitive and -resistant cell lines. The tumors of animals treated with IFN showed areas of necrosis, but this was not accompanied by infiltration of immune cells or vessel occlusion. In addition, they were unable to find evidence that the in vivo antitumor effect was mediated by cytotoxic cells which could be transferred between animals or inhibited by silica (which reportedly inhibits macrophages and to a lesser extent NK cells) nor could they demonstrate that other soluble factors were involved. In similar studies, Lee et al. (21) isolated an IFN-resistant cell line from IFN-sensitive L1210 leukemia cells and showed that this cell line, while resistant to IFN in vitro, remained sensitive to the antitumor effects of IFN in vivo. They concluded that host-mediated effects were critical for the antitumor effect of IFN. Our experiments also demonstrate that cells resistant to the in vitro antiproliferative effects of IFN remain sensitive to the in vivo antitumor effects of IFN; however, our results indicate that 3 of 3 independently isolated IFN-resistant clones (SIR-1, SIR-111, and SIR-E102) are significantly more sensitive than the parental cell line to the in vivo antitumor effects of IFN. These results provide compelling evidence that the enhanced in vivo response is a consequence of the defect in the IFN response pathway in the mutant cells and not due to an artifact of single cell cloning. The SIR-1 mutant is defective in IFN-induced antiviral and antiproliferative activity and is also defective in the induction of 2'-5'A synthetase, enhancement of H-2 antigen expression, and transient induction and subsequent repression of c-myc by IFN. The loss of control of one or more of these pathways or other specific genes regulated by IFN may result in a cellular phenotype that is more readily recognized by IFN-activated host defenses and eliminated.

The SIR-1 mutant has normal expression of H-2Kk antigens on its surface when compared to the parental cell line; however, it is unable to further increase this level of expression upon IFN treatment. Northern analysis demonstrates that nearly identical levels of mRNA for H-2 are produced in both the parental and mutant cell lines but that the accumulation of protein that occurs in the parental cell line following IFN treatment is abolished, suggesting that posttranscriptional pathways involved in regulating the stability of the H-2 protein are critical for IFN-mediated induction of H-2 antigens in these cells, and that this mechanism has been abolished in the SIR-1 cells.

Modulation of the major histocompatibility antigens has been associated with tumorigenicity (22). The evidence indicates that reduction or absence of Class I antigen expression reduces immune recognition, allowing cells to escape immune recognition and destruction. Many naturally occurring tumors lack or have significantly reduced expression of class 1 antigens, and the expression of a transfected class 1 gene in these cells can reverse tumorigenicity (23, 24). Class 1 expression can be repressed in adenovirus-transformed mouse cells, leading to increased tumorigenicity (25). Hayashi et al. (22) were able to demonstrate that IFN treatment of adenovirus 12-transformed cells could increase H-2 antigen expression and reduce tumorigenicity. Our findings indicate that constitutive expression of H-2 antigens is normal in the SIR-1 cells, but the IFN-mediated induction of H-2 antigen expression is abolished, and animals given injections of these cells have a small but statistically significant decrease in mean survival time without IFN treatment (18.1 versus 21.5 days). Furthermore, host-mediated events appear to be responsible for the enhanced antitumor effects of IFN against the IFN-resistant SIR-1 cells. These results argue that if the deregulation of H-2 antigen expression from IFN control is important to the enhanced in vivo therapeutic response to IFN, then the host cells may recognize the inability of cells to respond to IFN with a normal induction of H-2 antigens, or perhaps another IFN-regulated pathway, as abnormal, signalling that cell for elimination by host defenses.

The c-myc oncogene has been associated with a variety of lymphoid cancers (26, 27), and IFN has been shown to reduce the expression of c-myc in Daudi cells by a posttranscriptional (28, 29) or transcriptional (30) regulation. The 38C13 cells
express high levels of c-myc mRNA, and IFN has a biphasic effect on these cells; it transiently increases the abundance of c-myc mRNA within 1 h, followed by repression of c-myc mRNA to 10% of control levels by 24 h. SIR-1, while maintaining normal constitutive expression of c-myc mRNA, fails to repress levels of c-myc mRNA following IFN treatment. These results indicate that, like H-2 antigen regulation, constitutive levels of expression are not altered in the SIR-1 cell line, but interferon regulation is specifically abolished. While the exact function of c-myc in growth control is not understood, deregulation of c-myc occurs commonly in lymphoid neoplasms, and transgenic mice carrying c-myc linked to the immunoglobulin heavy chain enhancer have excessive proliferation of pre-B-cells and the development of B-cell malignancies (31, 32). Transformation of fibroblasts with various oncogenes including ras and EIA has resulted in increased sensitivity to host-mediated defenses including T-cells and NK cells. In addition, EIA has vestigial homology to c-myc (33); therefore, deregulation of c-myc expression from IFN control in this B-cell tumor, may result in a cellular phenotype that is more readily recognized by host immune systems and eliminated.

The results presented in this paper argue that resistance to the direct antiregulatory effects of IFN in vitro can occur at a high frequency and can result in the disruption of antiproiferative, antiviral, and immunomodulatory functions of IFN. In addition, in the three independently isolated cell lines, resistance to the in vitro antiregulatory effects of IFN results in increased sensitivity of the tumor cell lines to the in vivo antitumor effects of IFN, leading to an overall therapeutic advantage. These results indicate that, for this cell line, the antiregulatory effects of IFN are mediated by activation of host defenses and that resistance to the in vitro cytotoxic effects of IFN results in a tumor phenotype that is more readily recognized by host defenses and eliminated. The mechanism of the IFN-enhanced host-mediated response does not appear to require T-cell activity since the IFN-mediated response is intact in nude mice; however, NK cells appear to be required since the response is not observed in NK-defective beige mice.

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