Potentiation of the Cytocidal Effect of Human Immune Interferon by Different Synthetic Double-Stranded RNAs in the Refractory Human Colon Carcinoma Cell Line BE

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INTRODUCTION

IFNs are a group of natural proteins known to possess antiviral (1) as well as antitumor (2-4) properties. A comparison of the three types of IFNs revealed that IFN-α was a more potent growth inhibitor than either IFN-α or IFN-β in certain mouse (5) and human (6, 7) tumor systems and was toxic to a number of tumor cells in culture (8-11).

The synthetic dsRNA, poly(I)-poly(C), a potent IFN inducer, is active against a number of transplanted tumors (12, 13) and inhibited the growth of a variety of human tumor cells in culture (14, 15). A pronounced toxic response was observed when IFN-β-treated murine (16-18) and human (14, 19) cells were exposed to poly(I)-poly(C). In our previous report, treatment of IFN-γ-sensitive human colon carcinoma cell line HT-29 with IFN-γ and poly(I)-poly(C) resulted in a synergistic cytocidal effect (20).

In the present investigation we report the synergistic cytotoxic effect resulting from the combination of IFN-γ and poly(I)-poly(C) as well as other dsRNAs in the human colon carcinoma cell line BE which is inherently insensitive to the cytotoxic effects of IFN-γ and resistant to the toxic effects of dsRNAs. In addition, the relationship of this effect to the dsRNA-dependent (2',5')oligo(A)-Rnase L-pathway and rRNA transcription was examined.

MATERIALS AND METHODS

[methods-14C]Tryptophan (53 mCi/mmol), [2,8-3H]Adenosine (32 Ci/mmol), [3H]Uracil (140 Ci/mmol), and [3H]Thymidine (506 mCi/mmol) were purchased from New England Nuclear (Boston, MA), and [3H]leucine (506 mCi/mmol) was purchased from Amersham/Searle Corp. (Arlington Heights, IL). (2',5')oligo(A), poly(I)-poly(C)-agarose, poly(A)-poly(U), and poly(I)-poly(C)-poly(U) were purchased from Pharmacia-L. Biochemicals (Milwaukee, WI). Poly(ICLC) was kindly provided by Dr. Hilton Levy, National Institute of Arthritis and Infectious Diseases, Frederick, MD. The mismatched analogue of dsRNA, poly(I)-mismatched double-stranded RNA, where the C:U base ratio is 13:1; poly(ICLC), poly(A)-poly(U), or rI-rc(U13) and extracts were purchased from Millipore Filters (type HA; pore size, 0.45 μm). Incorporation of each precursor was expressed as dpm/106 cells.

(2',5')oligo(A) Synthetase Assay. Logarithmically growing cells in 25-cm2 flasks were incubated for 1 h with either 2 μCi of [3H]adenosine (0.5 Ci/mmol) plus 10-4 M 2'-deoxycoformycin, an adenosine deaminase inhibitor, and 1 μCi of [methyl-14C]thymidine (53 mCi/mmol) or 10 μCi of [3H]thymidine (2.6 mCi/mmol). Cold TCA-precipitable radioactivity was determined by liquid scintillation spectrometry. [3H]Thymidine incorporation was measured as hot (90°C for 10 min) TCA-precipitable radioactivity collected on Millipore filters (type HA; pore size, 0.45 μm). Incorporation of each precursor was expressed as dpm/106 cells.

(2',5')oligo(A) Synthetase Assay. Logarithmically growing cells in 175-cm2 flasks were exposed to IFN-γ and either poly(I)-poly(C), poly(ICLC), poly(A)-poly(U), or rI-rc(U13), and extracts were pre-
PARAPARED after lysing the cells with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4); 5 mM MgCl2; 120 mM KC1; 1 mM dihydrothreitol; 10% (v/v) glycerol; 0.5% Nonidet P-40. Cell extracts containing 21 µg of protein/10 µl were assayed for (2',5') oligo(A) synthetase activity as described previously (25). One unit of (2',5')oligo(A) synthetase is that amount which synthesizes 1 nmol of (2',5')oligo(A), per h at 30°C.

(2',5')Oligo(A) Levels in Intact Cells. Intracellular (2',5')oligo(A) levels were determined in logarithmically growing cells in 150-cm2 flasks following 1- or 3-day treatment with IFN-γ (100 units/ml) and/or poly(I)-poly(C) (100 µg/ml). Cells were harvested by trypsinization, washed with cold phosphate-buffered saline (6.3 mM Na2HPO4-0.8 mM KH2PO4-0.154 M), and extracted with 100 µl of TCA. Extracts were neutralized by shaking with 2 volumes of 0.5 M trictylamine in trifluorotrichloroethane. (2',5')Oligo(A) levels in neutralized extracts were measured by the radiobinding assay as described previously (8). Using our assay system, the minimum amount of (2',5')oligo(A) detectable was 20 fmol/106 cells.

RNA Extraction and Electrophoresis. Logarithmically growing cells in 175-cm2 flasks were prelabeled with 2.5 µCi of [3H]uridine and then treated with IFN-γ (100 units/ml) and either 100-µg/ml amounts of poly(I)-poly(C), poly(A)-poly(U), or rI-r(C13)U100, or 5-µg/ml amounts of poly(ICLC) for 3 days. Cells were pulse-labeled for 2 h with [3H]adenosine (1 µCi/ml; 0.5 Ci/mmol) and 10-4 M 2'-deoxycoformycin and harvested by scraping in cold phosphate-buffered saline. RNA was extracted and 0.2 unit was separated electrophoretically in composite gels containing 1.9% polyacrylamide:0.6% agarose:40 mM Tris-HCl (pH 7.6):20 mM sodium acetate:3 mM EDTA:10% glycerol as described previously (20).

RESULTS

Effect of IFN-γ and Poly(I)-Poly(C) on Cell Growth and Viability. Exponentially growing BE cells were treated with IFN-γ (10 to 300 units/ml) and/or poly(I)-poly(C) (100 µg/ml) for 3 days and cell growth and cell viability via a soft agar clonogenic assay were determined. IFN-γ (10 to 300 units/ml) or poly(I)-poly(C) (100 µg/ml) alone produced 10 to 20% growth inhibition, whereas the combination of the two drugs resulted in 60 to 80% growth inhibition (Fig. 1A). Similarly, IFN-γ (10 to 300 units/ml) resulted in 10 to 30% loss of cell viability and poly(I)-poly(C) (100 µg/ml) did not inhibit colony formation in these cells. In contrast, IFN-γ and poly(I)-poly(C) in combination produced 40 to 80% loss of cell viability after 3 days of continuous exposure (Fig. 1B). Incubation of cells with 400 neutralizing units of anti-IFN-α and anti-IFN-β antibodies during 3 days of treatment with IFN-γ and poly(I)-poly(C) did not inhibit the cytotoxicity indicating that potentiation of the toxicity of IFN-γ by poly(I)-poly(C) is not due to endogenous IFN secretion in dsRNA-treated cells (data not shown).

Fig. 1. Effect of IFN-γ and/or poly(I)-poly(C) on cell growth and viability. BE cells in log phase were treated for 3 days with IFN-γ and/or poly(I)-poly(C) and cell number (A) or colony formation (B) was determined. Each value is the mean of 3 experiments, where the standard error did not exceed 5%.

Fig. 2. Effect of IFN-γ and poly(I)-poly(C), poly(A)-poly(U), poly(ICLC), or rI-r(C13)U100 on Cell Growth and Viability. Logarithmically growing BE cells were exposed to IFN-γ (100 units/ml) and/or varying concentrations of poly(I)-poly(C), poly(A)-poly(U), poly(ICLC), or rI-r(C13)U100, for 3 days and cell growth and cell viability was determined as a function of the concentration of the dsRNA. IFN-γ alone produced 20% growth inhibition and 2.5- to 100-µg/ml amounts of poly(I)-poly(C), poly(A)-poly(U), or rI-r(C13)U100, or 25- to 25-µg/ml amounts of poly(ICLC) reduced cell growth by 5 to 20%. However, IFN-γ, in combination with varying concentrations of dsRNA, produced a 30 to 80% reduction in cell growth (Fig. 2A). Marginal loss of cell viability was observed after exposure of these cells to similar regimes of either IFN-γ or the various dsRNAs (Fig. 2B). The combination of IFN-γ with each dsRNA resulted in a synergistic cytocidal effect, which produced a 40 to 95% reduction in cell viability over a dsRNA dose range of 2.5 to 100 µg/ml (Fig. 2B). In combination with IFN-γ (100 units/ml), the concentration of dsRNA producing a 50% reduction in viability was 6 µg/ml for poly(I)-poly(C), 1 µg/ml for poly(A)-poly(U), 3 ng/ml for poly(ICLC), and 16 µg/ml for rI-r(C13)U100.

DNA, RNA, and Protein Synthesis in Cells Treated with IFN-γ and Poly(I)-Poly(C). Cells were pulse-labeled with [3H]thymidine, [3H]adenosine, and [3H]leucine as measures of DNA, RNA, and protein synthesis, respectively. [3H]Adenosine was selected over [3H]uridine as a measure of RNA synthesis since the specific activity of intracellular [3H]UTP was significantly lowered in poly(I)-poly(C)-treated cells pulse-labeled with [3H]uridine in comparison to untreated cells. This effect was due to the breakdown of the C strand of poly(I)-poly(C) by intracellular nucleases resulting in higher levels of cytidine and subsequent inhibition of uridine phosphorylation. The specific activ...
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Reduced DNA synthesis after 3 days of treatment with IFN-γ and poly(I)-poly(C) paralleled growth inhibition (Fig. 3). Three days of treatment with IFN-γ (100 μg/ml) and poly(I)-poly(C) (100 μg/ml) reduced DNA, RNA, and protein synthesis by 75, 50, and 30%, respectively.

(2',5')Oligo(A) Levels and (2',5')Oligo(A) Synthetase Activity in Cells Treated with IFN-γ and dsRNA. To determine if the dsRNA-activated (2',5')oligo(A)-RNase L-pathway was involved in the cytotoxic effect of the combination of IFN-γ and dsRNA, (2',5')oligo(A) synthetase activity was measured in cells treated with IFN-γ and either poly(I)-poly(C), poly(A)-poly(U), poly(ICLC), or rI-r(C13,U) (Table 1). A 1-day exposure to IFN-γ (100 units/ml) resulted in a 4-fold induction of (2',5')oligo(A) synthetase activity and 100-μg/ml amounts of poly(I)-poly(C), poly(A)-poly(U), or rI-r(C13,U) (Table 1). A 1-day exposure to poly(I)-poly(C), poly(A)-poly(U), or rI-r(C13,U) resulted in 1.2- to 1.6-fold induction of (2',5')oligo(A) synthetase activity. The combination of IFN-γ with any of the dsRNAs under study failed to show any further elevation of (2',5')oligo(A) synthetase activity. Moreover, no measurable amount of (2',5')oligo(A) was detected by the radioligand assay in extracts of BE cells after 1 or 3 days of treatment with IFN-γ and poly(I)-poly(C), despite the ability of this method to detect concentrations as low as 20 fmol of (2',5')oligo(A)/10⁶ cells (results not shown).

DISCUSSION

The present study has examined the comparative effects of a variety of synthetic dsRNAs in combination with IFN-γ in a human undifferentiated colon carcinoma cell line. BE cells are resistant to the growth-inhibitory or cytotoxic effects of poly(I)-poly(C), poly(A)-poly(U), poly(ICLC), or the mismatched dsRNA, rI-r(C13,U), and exhibited only a slight response to the cytotoxic effects of IFN-γ. However, each of the dsRNAs examined when used in combination with IFN-γ generated a synergistic cytoidal response. These data suggest that tumor cells inherently resistant to IFN might respond to such a combination treatment regimen.

Although all dsRNAs under study were capable of potentiating the toxic effect of IFN-γ, the optimal concentration for individual dsRNA differed to a significant extent. A 2000-fold reduced the specific activity of [3H]adenosine into 28S and 18S rRNA after 3 days of treatment (Fig. 4). Moreover, no degradation of rRNA prelabeled with [14C]uridine or pulse-labeled with [3H]adenosine was observed after treatment with IFN-γ and poly(I)-poly(C) (Fig. 4). No effect of IFN-γ and poly(A)-poly(U), poly(ICLC), or rI-r(C13,U) on rRNA transcription was observed in these cells (results not shown).

Table 1 (2',5')Oligo(A) synthetase activity following treatment of HT-29 cells with IFN-γ and poly(I)-poly(C), poly(A)-poly(U), poly(ICLC), or rI-r(C13,U).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(2',5')Oligo(A) synthetase activity (units/mg)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.0 ± 0.6 *</td>
<td>1.0</td>
</tr>
<tr>
<td>Poly(I)-poly(C), 100 μg/ml</td>
<td>4.7 ± 0.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Poly(A)-poly(U), 100 μg/ml</td>
<td>3.7 ± 0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Poly(ICLC), 5 μg/ml</td>
<td>3.7 ± 0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>rI-r(C13,U), 100 μg/ml</td>
<td>3.6 ± 0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>IFN-γ, 100 units/ml</td>
<td>11.9 ± 1.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Poly(I)-poly(C), 100 μg/ml</td>
<td>13.1 ± 1.2</td>
<td>4.4</td>
</tr>
<tr>
<td>IFN-γ, 100 units/ml</td>
<td>13.0 ± 2.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Poly(A)-poly(U), 100 μg/ml +</td>
<td>10.6 ± 0.6</td>
<td>3.5</td>
</tr>
<tr>
<td>IFN-γ, 100 units/ml</td>
<td>14.9 ± 2.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Poly(ICLC), 5 μg/ml + IFN-γ, 100</td>
<td></td>
<td></td>
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<tr>
<td>units/ml</td>
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* Mean ± SD of duplicate assays from 2 experiments.
higher concentration of poly(I)·poly(C) (LC50 = 6 μg/ml) in comparison to poly(I-ICLC) (LC50 = 3 ng/ml) was required to generate an equivalent cytotoxic response. It is possible that such a difference is related to the increased resistance of poly(I-ICLC) to hydrolysis by pancreatic RNase in comparison to the parent poly(I)-poly(C) (26). Since poly(I-ICLC) is an effective IFN inducer in primates (26) and is currently undergoing clinical trials (27), our data suggest that its use as an adjuvant to IFN therapy may be beneficial. On the contrary, a 3-fold higher concentration of the mismatched dsRNA, rI·r(C12,U)n, (LC50 = 16 μg/ml) than poly(I)-poly(C) was needed to reduce cell viability by 50%. This may be attributable to the increased sensitivity of rI·r(C12,U)n to RNase (28). The mismatched dsRNA, rI·r(C12,U)n, which has an antiviral activity similar to that of rI·r(C13,U)n (21), was shown to be a less effective antiproliferative agent than poly(I)-poly(C) in six human tumor cell lines (15). In combination with IFN-β, rI·r(C12,U)n produced an additive growth-inhibitory effect in human bladder carcinoma cell line RT-4 (29). On the other hand, Rosenblum and Gutterman (30) reported synergistic antiproliferative activity in human melanoma cells treated with IFN-α and mismatched dsRNA. The present study indicates that the combination of IFN-γ and mismatched dsRNA produces a synergistic cytotoxic effect in tumor cells which are resistant to mismatched dsRNA or IFN-γ alone. This observation might be important since rI·r(C12,U)n is undergoing clinical trials (31).

Comparison of the potentiating abilities of poly(I)·poly(C) and poly(A)·poly(U) revealed that a 4-fold higher concentration of poly(I)·poly(C) than poly(A)·poly(U) (LC50 = 1 μg/ml) was needed to reduce the viability of cells by 50%. Poly(A)·poly(U) has been shown previously to be a poor interferon inducer (32) and was less toxic than poly(I)·poly(C) to mouse L929 cells pretreated with IFN-β (33). However, poly(A)·poly(U) has been successful as an adjuvant to radiation therapy in patients with operable breast cancer (34, 35). Our results also suggest that poly(A)·poly(U) may be potentially useful as an adjuvant to IFN-γ therapy.

Several investigators have examined the mechanisms underlying poly(I)·poly(C) toxicity in IFN-treated cells. Stewart et al. (18) observed poly(I)·poly(C) toxicity in cells treated with IFN-β despite the presence of actinomycin D or cycloheximide in the culture medium. These results indicated that transcriptional and posttranscriptional processes were not involved. Wallach and Revel (19) reported that the cytolytic effect of poly(I)·poly(C) in human foreskin fibroblasts treated with IFN-β was significantly inhibited by dexamethasone, suggesting that the mechanism of cytosis may be related to the inhibition of prostaglandin biosynthesis. Several investigators have examined the dsRNA-dependent (2',5')oligo(A)-RNase L-pathway in IFN- and poly(I)-poly(C)-treated cells. Nilsen et al. (36) reported elevated (2',5')oligo(A) levels in HeLa cells treated with IFN-β and poly(I)-poly(C). Goswami and Sharma (37) observed increased (2',5')oligo(A) levels in IFN-β- and poly(I)·poly(C)-treated mouse L-cells. However, Faure et al. (38) observed identical (2',5')oligo(A) synthetase and protein kinase induction in mouse fibroblasts sensitive and resistant to treatment with IFN-β and poly(I)-poly(C). In our previous report (20), a synergistic effect was noted by the combination of IFN-γ and poly(I)-poly(C) on the induction of (2',5')oligo(A) synthetase activity in HT-29 cells despite the observation that endogenous (2',5')oligo(A) was not detectable in these cells. In the present study, IFN-γ induced (2',5')oligo(A) synthetase activity in BE cells to an extent similar to that in HT-29 cells (20) but the activity was not increased further after combination treatment with IFN-γ and poly(I)-poly(C). Moreover, neither (2',5')oligo(A) nor RNA degradation were observed in BE cells treated with IFN-γ and poly(I)-poly(C). These results suggest a lack of involvement of the dsRNA-dependent (2',5')oligo(A)-RNase L-pathway in the cytotoxicity mediated by IFN-γ and poly(I)-poly(C). In addition, dsRNA-dependent protein kinase activity was not induced after treatment with IFN-γ and poly(I)-poly(C) (results not shown) eliminating this as a mechanism of action of IFN-γ and poly(I)-poly(C) in BE cells. In HT-29 cells treated with IFN-γ and poly(I)-poly(C), the only event which correlated with the onset of toxicity was the inhibition of rRNA transcription (20). However, in BE cells, rRNA transcription was not inhibited after treatment with IFN-γ and poly(I)-poly(C). Thus, the mechanism responsible for the antitumor effects of IFN-γ and dsRNA in these cell lines still remains obscure. Further studies exploring other possible modes to their action such as their effects on cellular oncogenes and cell cycle progression are in progress.

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POTENTIATION OF CYTOCIDAL EFFECTS OF IFN-γ

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