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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ABSTRACT

A human cell line BE, derived from an undifferentiated carcinoma of the colon, was studied for its response to the cytocidal effects of human immune interferon (IFN-γ) alone and in combination with various double-stranded RNAs (dsRNAs). BE cells were moderately refractory to 3-day treatment with IFN-γ (10 to 300 units/ml) where only 5 to 30% reduction in colony formation occurred. A similar exposure interval to polyriboinosinic-polyribocytidylic acid [poly(I)-poly(C)] (100 µg/ml) had no detectable effect on colony formation. In contrast, the lethal effect of the combination of IFN-γ and poly(I)-poly(C) was synergistic and this regimen produced a 40 to 80% reduction in colony formation. The cytocidal effects of the combination of IFN-γ with varying concentrations of the dsRNAs poly(I)-poly(C), polyriboadenyllic-polyribouridylic acid [poly(A)-poly(U)], polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine in carboxymethylcellulose [poly(IICLC)], and mismatched dsRNA [rL-r(C13,U13)] were also examined. The concentration of the dsRNAs producing a 50% decrease in cell viability in combination with IFN-γ (100 units/ml) was 6 µg/ml for poly(I)-poly(C), 1 µg/ml for poly(A)-poly(U), 3 ng/ml for poly(IICLC), and 16 µg/ml for rL-r(C13,U13). DNA, RNA, and protein synthesis in IFN-γ and poly(I)-poly(C)-treated cells were reduced in a dose-dependent manner. However, there were no changes in either (2',5')oligoadenylate concentrations or in ribosomal RNA transcription following treatment with IFN-γ and poly(I)-poly(C). Thus, the synergism resulting from the combination of IFN-γ and dsRNA appears to be mediated via another, as yet unknown, mechanism.

INTRODUCTION

IFNs1 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-γ 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 cytotoxic 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

Materials

[2H]Thymidine (53 mCi/mmol), [3H]Leucine (140 Ci/mmol), and [14C]Thymidine (53 mCi/mmol) were purchased from New England Nuclear (Boston, MA), and pppA(2'pA)3[32P]pCp (3000 Ci/mmol) was purchased from Amer sham/Seearle Corp. (Arlington Heights, IL). (2',5')Oligo(A), poly(I)-poly(C)-agarose, poly(A)-poly(U), and poly(I)-poly(C)-treated cells were purchased from Pharmacia-P. L. Biochemicals (Milwaukee, WI). Poly(IICLC) was kindly provided by Dr. Hilton Levy, National Institute of Arthritis and Infectious Diseases, Frederick, MD. The mismatched analogue of dsRNA, rL-r(C13,U13), was synthesized according to the method described by Ts'o et al. (21). The C.U ratio was determined by digestion of 50 nmol of polyribonucleotide with RNase A (1 mg/ml) in 30 µl of 10 mM Tris-Cl (pH 7.4):10 mM EDTA:0.2 mM NaCl for 2 h at 37°C. The digest was further digested to ribonucleosides with snake venom phosphodiesterase and bacterial alkalase phosphatase, and cytidine and uridine were separated by reverse-phase high performance liquid chromatography as described previously (22). IFN-γ (100 units/mg) was purchased from Cellular Products (Buffalo, NY).

Cells and Incubation.

BE cells originally derived from an undifferentiated human colon carcinoma (23) were grown under an atmosphere of air and 5% CO2 in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4), and gentamicin (50 µg/ml). Cell inocula were 102/101 ml in 25-cm2 flasks and 103/100 ml in 175-cm2 flasks.

Colony Formation and Cell Growth.

Cell viability was determined by the soft agar clonogenic assay as described previously (24). Briefly, cells were washed with Hanks' balanced salt solution, without Ca2+ or Mg2+, and containing 20 mM EDTA, and trypsinized with 0.1% trypsin in Hanks' balanced salt solution at 37°C for 10 min. Cells were then plated at concentrations of 100 to 1000 cells/ml of RPMI 1640 containing 20% fetal calf serum and 0.2% agar. Colonies were counted after 14 days. Loss of cell viability is defined as the inability of a single cell to form a colony after 14 days of incubation in drug-free soft agar medium. At the time of plating, greater than 90% of control or drug-treated cells excluded trypan blue. Cell growth was monitored by counting cells with a Model ZM Coulter Counter.

DNA, RNA, and Protein Synthesis.

Logarithmically growing cells in 25-cm2 flasks were incubated for 1 h with either 2 µCi of [3H]Thymidine (0.5 Ci/mmol) plus 10-4 M 2'-deoxycoformycin, an adenosine deaminase inhibitor, and 1 µCi of [2H]Thymidine (53 mCi/mmol) or 10 µCi of [3H]Leucine (2.6 mCi/mmol). Cold TCA-precipitable radioactivity was collected on glass fiber discs and 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/104 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(IICLC), poly(A)-poly(U), or rL-r(C13,U13), and extracts were pre-

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2 The abbreviations used are: IFN, interferon; dsRNA, double-stranded RNA; IFN-α, leukocyte interferon; IFN-β, fibroblast interferon; IFN-γ, immune interferon; poly(I)-poly(C), polyriboinosinic-polyribocytidylic acid; poly(A)-poly(U), polyriboadenyllic-polyribouridylic acid; poly(IICLC), polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine in carboxymethylcellulose; rL-r(C13,U13), mismatched double-stranded RNA, where the C:U base ratio is 1:3; (2',5')oligo(A), pppA(2'pA)n; TCA, trichloroacetic acid; LC50, concentration producing a 50% reduction in cell viability.
pared after lysing the cells with 20 mM 4-(2-hydroxyethyl)-1-piperazin-ethanesulfonic acid (pH 7.4):5 mM MgCl₂:120 mM KC₁:1 mM dithiothreitol: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)ₙ 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 mM MgCl₂-0.154 mM KCl) and extracted with 100 μl of TCA. Extracts were neutralized by shaking with 2 volumes of 0.5 M trioctylamine 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 [³²P]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 rLₐ-r(C₁₃)Uₙ, 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 μl 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).

Comparative Effects of the Combinations of IFN-γ and Poly(I)-Poly(C), Poly(A)-Poly(U), Poly(ICLC), or rLₐ-r(C₁₃)Uₙ 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 rLₐ-r(C₁₃)Uₙ, 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 rLₐ-r(C₁₃)Uₙ, or 25- to 250-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 regimens of either IFN-γ or the various dsRNAs (Fig. 2B). The combination of IFN-γ with each dsRNA resulted in a synergistic cytotoxic 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 rLₐ-r(C₁₃)Uₙ.

DNA, RNA, and Protein Synthesis in Cells Treated with IFN-γ and Poly(I)-Poly(C). Cells were pulse-labeled with [³²P]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 activi-

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 rLₐ-r(C₁₃)Uₙ on cell growth and viability. BE cells in log phase were treated for 3 days with IFN-γ and poly(I)-poly(C), poly(A)-poly(U), poly(ICLC), or rLₐ-r(C₁₃)Uₙ, 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%.
potentiation of cytotoxic effects of IFN-γ

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)3, 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 cytotoxic 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 reduction in specific activity of [3H]adenosine into 28S and 18S rRNA after 3 days of treatment (Fig. 4). Moreover, no degradation of rRNA prelabeled with [3H]adenosine 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)3 on rRNA transcription was observed in these 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)3, 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 cytotoxic 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
higher concentration of poly(I)-poly(C) (LC50 = 6 μg/ml) in comparison to poly(I:CLC) (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:CLC) to hydrolysis by pancreatic RNase in comparison to the parent poly(I)-poly(C) (26). Since poly(I:CLC) 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, rIα-r(Ci3,U)α (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 rIα-r(Ci3,U)α to RNase (28). The mismatched dsRNA, rIα-r(Ci3,U)α, which has an antiviral activity similar to that of rIα-r(Ci3,U)α (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, rIα-r(Ci3,U)α 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 rIα-r(Ci3,U)α 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). 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) and poly(A)-poly(U) compounds (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.

REFERENCES

POTENTIATION OF CYTOTOXIC EFFECTS OF IFN-γ

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