Antitumor Effects of Polyribonucleotides for Mouse Transitional Cell Carcinoma Enhanced by Cyclophosphamide

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ABSTRACT

Mouse bladder tumor (MBT-2), derived from a carcinogen-induced transitional cell carcinoma of the bladder, has proven a useful model for study of pathogenesis and prediction of cytotoxic drug sensitivity of human bladder carcinoma. To define optimal conditions for activity of the potent Interferon inducer polyriboinosinic-polyribocytidylic acid [poly(l)-poly(C)] in this model, studies of dose, timing, and combinations with a cytotoxic drug were initiated. Poly(l)-poly(C) inhibited MBT-2 growth when 10^6 or 10^7 tumor cells were implanted. Tumor growth reduction was relatively more pronounced in mice inoculated with higher numbers of MBT-2 cells (10^7) than in mice inoculated with an intermediate dose (10^6) or small dose (10^5). In mice inoculated with 10^6 MBT-2 tumor cells, poly(l)-poly(C) (2.5 or 10 mg/kg i.p.) on Days 5 to 19 every other day reduced tumor size markedly. It had no effect, however, on tumor incidence or the time of their first detection. Treatment for a shorter period (alternate days from Days 11 to 19) resulted in less inhibition of tumor growth. Once treatment was discontinued, tumors grew progressively. Polyriboadenylic:polyribouridylic acid [poly(A)-poly(U)] (10 mg/kg) which inhibited tumor growth but to a lesser degree than poly(l)-poly(C) induced lower, less sustained levels of serum interferon. Cyclophosphamide, injected i.p. on Day 1, resulted in inhibition of tumor incidence and growth in direct proportion to the dose administered (25 to 200 mg/kg), but it was curative only at ≥30% lethal doses. When combined with poly(l)-poly(C) (2.5 or 10 mg/kg), cyclophosphamide (50 mg/kg) had an additive antitumor effect. Optimal inhibition of MBT-2 tumor growth occurred by combining cyclophosphamide (100 mg/kg) with poly(l)-poly(C) (2.5 mg/kg); eight of 14 mice were tumor free on Day 60.

INTRODUCTION

Polyribonucleotides, such as poly(l)-poly(C) and poly(A)-poly(U), either alone or in combination with various polycations, remain among the most effective synthetic inducers of IFN. Polyribonucleotides have proven effective against a broad range of experimental tumors of spontaneous, carcinogen, and viral origin (11, 14, 16, 21). Effectiveness of polyribonucleotides has varied as a function of tumor and duration of treatment. IFNs are therapeutically effective in many experimental animal models (3, 4, 8–10). The roles of IFNs and IFN inducers in treatment of bladder carcinoma could potentially be elucidated by use of the murine experimental model, which has a well-defined etiology and pathogenesis (7). Cytotoxic drugs active in animal models, including those active in transitional cell carcinoma, have often proven effective in clinical trials (13, 23).

The objectives of these studies were: (a) to determine conditions for effectiveness of poly(l)-poly(C) and poly(A)-poly(U) in a transplatable transitional cell carcinoma of the mouse bladder, MBT-2, originally derived by induction by the chemical carcinogen FANFT; and (b) to determine effects in this model of polyribonucleotides when combined with a cytotoxic drug.

MATERIALS AND METHODS

Mice. Five- to 6-week-old female C3H/He mice purchased from Sprague-Dawley (Indianapolis, IN) were housed in suspended metal cages with no more than 5 mice/cage. They were fed Wayne Rodent Blox (Continental Grain Co., Chicago, IL) and HCI-acidified water ad libitum and were placed in rooms with controlled temperature (22.2–24.4°C), humidity (40%), and 12-hr light-dark cycles. Mice, used at least 1 week after their arrival, were weighed on Day 1 after tumor cell inoculation and then every other day until the end of each experiment. No differences occurred in mouse weight between groups except as related to tumor size.

Tumors. MBT-2 (24), derived from a FANFT-induced bladder carcinoma provided by Dr. Mark Soloway (University of Tennessee, Memphis, TN), was maintained in vivo by s.c. transfer to C3H mice every 2 weeks. Nonneoplastic parts of the tumor were dissociated using a 0.05% trypsin-0.02% EDTA preparation for 2 to 5 min. Tumor cells were then washed twice in Hanks' balanced salt solution and suspended in the desired concentration of viable cells. Cells were inoculated in 0.1-ml volume s.c. in the right inguinal region in all the mice except in experiments designed to test for regional differences in tumor growth (1), where tumor cells were also inoculated in the anterior lateral trunk region. Usually a group consisted of 15 mice.

Tumor growth was assessed by measuring with a caliper the longest dimension and a dimension perpendicular to it. A formula to quantify tumor size, namely (Diameter 1) (Diameter 2) (0.523), was applied (12). Measurements were made every other day until Day 21, at which time mice with tumors were killed, and their tumors were measured, dissected, and weighed. Mice with very small tumors and those free of palpable tumors on Day 21 were kept alive, and tumors were measured every other day until Day 40. Mice were then killed, and tumors were measured, dissected, and weighed. In all groups, mean tumor weights were based on mice which developed tumors.

Drugs. CY (Mead Johnson, Evansville, IN) was prepared fresh and injected i.p. at the desired concentration and the specified time after tumor cell inoculation.

Poly(l)-poly(C) (Lot 124723) and poly(A)-poly(U) (Lot 845211) were purchased from PL Biochemicals, Milwaukee, WI. Each was weighed and allowed to dissolve in phosphate-buffered saline at 4°C overnight for
a final concentration of 1 mg/ml. Poly(l)-poly(C) was reannealed by heating in a 70° water bath for 10 min, and the contents were mixed and then returned to a 70° bath for 5 min. The water bath was then allowed to cool gradually overnight. The solution was then aliquoted and frozen. Poly(A)-poly(U) was stored at 4°. The drugs were injected i.p. at the specified time and dose.

Dose choice was determined by studies of toxicity of CY and poly(l)-poly(C). IFN levels were measured in the sera of mice given injections of different doses of poly(l)-poly(C) or poly(A)-poly(U). Antiviral activity of the pooled sera was determined in an encephalomyocarditis virus hemagglutinin yield-reduction assay utilizing L929 target cells (20). Titers are derived graphically from the 0.5 log (1.66 log5) intercept on a plot of the viral yields against the IFN dilution. Units were adjusted using National Institute of Allergy and Infectious Diseases Mouse Reference Interferon G-002-904-511.

**In Vitro Studies.** For in vitro studies, MBT-2 cells were grown as monolayers in 75-sq cm polystyrene culture flasks (Corning, NY). The growth medium used was Eagle’s minimal essential medium with Earle’s salts (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.,) 2 mM glutamine, and gentamicin (50 μg/ml). Cells were subcultured 1 to 2 times weekly using trypsin (0.05%)-EDTA (0.02%) (Grand Island Biological Co.). Cells were grown and assayed in a 37° 5% CO2 humidified, incubator.

Colony formation experiments were set up by adding 100 to 200 cells to 60- x 15-mm plastic Petri dishes. Triplicate plates were treated with IFN or minimal essential medium and allowed to incubate for 10 days, at which time, plates were fixed with 95% ethanol and stained with 0.2% methylene blue. Colony number was quantitated using a plaque viewer (Bélico, Vineland, NJ). Only colonies with greater than 50 cells were counted.

Murine lymphoblastoid IFN was a gift from Dr. I. Grosser of the Institut de Recherches Scientifique sur le Cancer, Villejuif, France. It was induced in C-243 cells with Newcastle disease virus (15).

**RESULTS**

**Conditions for Effectiveness of Poly(l)-poly(C) and Poly(A)-poly(U).** Inoculation of 10^6 cells anteriorly into the upper lateral trunk region resulted in a significantly larger tumor than when the same number of cells were injected posteriorly at the inguinal area (Chart 1). The differences were apparent by Day 13 and then became more pronounced and significant on Days 15, 17, and 19 (p < 0.01 for all 3 days). Although the relative decrease at the anterior site was greater, final tumor size at both sites was equivalent after poly(l)-poly(C) (10 mg/kg) treatment (Chart 1). Tumor cells were routinely inoculated in the inguinal region in the remaining experiments.

When given continuously on an alternate day schedule, different doses of poly(l)-poly(C) caused varied effects (Chart 1). A low dose of 0.1 mg/kg enhanced tumor growth in mice inoculated with 10^6 cells. Poly(l)-poly(C) (1 mg/kg) caused a slight reduction in tumor size in mice inoculated with 10^6 cells. Inhibition consistently occurred at 10 mg/kg. When poly(l)-poly(C) was administered for only 3 doses on alternate days beginning 1 day after MBT-2 cell inoculation, only slight and not significant inhibition of tumor size occurred (data not shown).

Inhibition of MBT-2 tumor growth after treatment with poly(l)-poly(C) was affected by the number of cells inoculated. When 10^6 cells were inoculated, tumor incidences were very low. Only 1 of 15 mice in the treated and untreated groups had palpable tumors at Day 21. When 10^7 MBT-2 cells were inoculated, about 66% of both treated and untreated hosts developed tumors. At this cell dose, neither tumor weight nor tumor size at Day 21 was significantly different in the poly(l)-poly(C)-treated (10 mg/kg at Days 1 to 19 every other day) and untreated mice. When 10^5 or 10^6 cells were inoculated, all mice developed palpable tumors about Day 9 after implantation. Tumors in untreated mice were significantly larger than those in mice treated with poly(l)-poly(C) (10 mg/kg) (on alternate days from Days 1 to 19). The difference in tumor dimensions and weight between treated and untreated mice was more pronounced when 10^6 rather than 10^5 cells were inoculated (Table 1).

To determine whether poly(l)-poly(C) or poly(A)-poly(U) would inhibit established tumors, alternate-day injections were initiated either 5 or 11 days after tumor cell inoculation. Tumor growth was inhibited when poly(l)-poly(C) or poly(A)-poly(U) (10 mg/kg) was begun either during a prepayable (Day 5) or palpable, clinical (Day 11) stage (Table 2). Poly(l)-poly(C) or poly(A)-poly(U) (10 mg/kg) had no effect, however, on the frequency of tumors. When 10^5 MBT-2 tumor cells were inoculated s.c. and poly(l)-poly(C) was administered every other day from Day 5 or Day 11, all mice developed tumors at the same time as the controls (Table 2). Alternate-day administration of 2.5 mg/kg was effective
but less than 10 mg/kg of poly(l)-poly(C) (Table 2). As long as poly(l)-poly(C) was administered every other day, tumor size and tumor weight remained suppressed (Table 2). However, once treatment stopped, tumors grew progressively. No curative effects occurred with poly(l)-poly(C) alone.

Poly(A).poly(U) (10 mg/kg) caused a significant (p < 0.01) reduction in tumor size when compared to the growth of MBT-2 in untreated mice (Table 2). However, antitumor effects of poly(A)-poly(U) were less than effects of poly(l)-poly(C).

**IFN Induction and Antiproliferative Effects in Vitro.** The effect of poly(l)-poly(C) could result from induction of IFN. To test this, C3H mice were bled 6 and 24 hr after i.p. injection of different doses of poly(l)-poly(C) and poly(A)-poly(U), and the serum was assayed for IFN. Injections of poly(l)-poly(C) resulted in high levels of serum IFN at 6 hr (Table 3). Persistent IFN was present in the serum at 24 hr after poly(l)-poly(C). Low levels of IFN were found in the sera of mice treated with poly(A)-poly(U).

At 500 units/ml, levels well below those measured in poly(l)-poly(C)-treated mice, mouse IFN inhibited colony formation by MBT-2 in vitro (Table 4), p < 0.01.

**Combination of CY with Poly(l)-poly(C).** When combined with different concentrations of CY, poly(l)-poly(C) (10 mg/kg) was lethal. The time of administration and dose of CY, together with the time of injection and dose of poly(l)-poly(C), affected the severity of the toxicity (Table 5). Death occurred within 24 hr...
The optimally effective combination was CY (100 mg/kg) on Day 1 followed by poly(l)-poly(C) (2.5 mg/kg) on alternate days on Days 5 to 19 (Chart 4). This regimen caused no body weight change, and all mice survived the treatment. On Day 21, only one of 14 (Chart 4) mice had very small, hardly palpable tumors. On Day 40, 6 of 14 mice had measurable tumors. All mice were followed to Day 60. No further tumors appeared in the remaining 8 mice. Thus, 8 of 14 mice were "cured" by the treatment.

The 8 mice which remained tumor free on Day 40 were further inoculated on that day with $1 \times 10^6$ MBT-2 cells on the left side of the mice in corresponding sites to the previous tumor cell inoculation. The same inoculum was introduced to fresh mice of the same age. The tumor resulting from the second inoculation was significantly ($p < 0.05$) smaller in the "cured" mice (1.14 ± 0.21 cu cm) than in the fresh mice (2.07 ± 0.32 cu cm).

**DISCUSSION**

Treatment with polyribonucleotides resulted in significant inhibition of growth of transplantable, transitional cell carcinoma of the bladder in histocompatible mice. Variables affecting this result included location and size of tumor inoculum together with schedule, dose, and type of polyribonucleotide. MBT-2 grew at an expected accelerated rate (2) when the cells were inoculated in the anterior lateral trunk region as compared to the same number of cells inoculated posteriorly in the inguinal area, possibly as a result of a more efficient anterior site microvasculature (1). In mice treated with poly(l)-poly(C), tumor size in both locations was comparable, suggesting greater relative effectiveness of the treatment at the anterior sites.

The number of implanted cells also influenced effectiveness of polyribonucleotides. At a cell number which initiated tumors in only 66% of the inoculated mice ($10^6$ MBT-2 cells), treatment with poly(l)-poly(C) had no effect on tumor size or tumor incidence. Significant inhibition, however, occurred after treatment with poly(l)-poly(C) when $10^5$ and more so when $10^6$ cells were inoculated. This suggests effectiveness of treatment with poly(l)-poly(C) increased with larger tumor inocula. Polyribonucleotides were effective regardless of whether treatment was initiated shortly after tumor cell implantation (24 or 120 hr later) or whether begun when tumors were already palpable. A greater proportional inhibition occurred, however, with more prolonged (8 or 10 doses) duration of administration when compared to a more limited duration (5 doses on alternate days). Lower doses of poly(l)-poly(C) were either ineffectual or caused a slight enhancement of MBT-2 growth. Poly(A)-poly(U) was also significantly inhibitory of MBT-2 tumor growth but was less effective than the same concentration of poly(l)-poly(C). Despite the significant inhibitory effects of the 2 polyribonucleotides on MBT-2 tumor size, inhibition of tumor growth occurred only as long as the drugs were administered. When treatment was discontinued, tumors grew without inhibition.

Noncurative, tumor-suppressive effects of polyribonucleotides have been the common occurrence in experimental tumor models (11, 14, 16, 21). Augmented inhibition of tumor growth with about 60% of mice being cured of implanted MBT-2 tumors resulted from combined use of poly(l)-poly(C) with CY. Both drugs were utilized at doses which were nonadditive in toxicity. Although the combination of polyribonucleotides with cytotoxic drugs has been reported previously to be additive, cures have
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Chart 3. A, effects on MBT-2 tumor growth (10^6 cells inoculated s.c. on Day 0) of treatment with CY (25 mg/kg) alone on Day 1 or in combination with 2.5 or 10 mg poly(I)-poly(C) per kg on Days 5 to 19 on alternate days. When compared to Chart 3B, it is clear that there are no additive effects. □, untreated; ○, CY (25 mg/kg); △, CY (25 mg/kg) plus poly(I)-poly(C) (2.5 mg/kg); •, CY (25 mg/kg) plus poly(I)-poly(C) (10 mg/kg). B, effects on MBT-2 tumor growth (10^6 cells inoculated s.c. on Day 0) of poly(I)-poly(C) (2.5 or 10 mg/kg) on Days 5 to 19 on alternate days. □, untreated; •, poly(I)-poly(C) (2.5 mg/kg); ○, poly(I)-poly(C) (10 mg/kg). C, effects on MBT-2 tumor growth (10^6 cells inoculated s.c. on Day 0) of CY (50 mg/kg) on Day 1 alone or in combination with poly(I)-poly(C) (2.5 or 10 mg/kg) on Days 5 to 19 on alternate days. □, untreated; •, CY (50 mg/kg); ○, CY (50 mg/kg) plus poly(I)-poly(C) (2.5 mg/kg); △, CY (50 mg/kg) plus poly(I)-poly(C) (10 mg/kg).

only rarely been reported (26). Poly(I)-poly(C) has been reported recently to protect mice from 5-fluorouracil toxicity (25); antitumor effectiveness of the combination has not yet been reported.

The level of IFN induced in mouse sera following poly(I)-poly(C) administration suggest IFN production may be an important factor in the inhibition of MBT-2 tumor growth. Furthermore, concentrations of mouse IFN, 10- to 20-fold less than peak serum levels, inhibited MBT-2 cell proliferation in vitro. IFN, however, may not be the only factor in antitumor effectiveness of polyribonucleotides (12). Poly(I)-poly(C) (0.1 mg/kg) and poly(A)-poly(U) (10 mg/kg) resulted in almost identical peak levels of IFN production (320 units/ml serum at 6 hr). The former, however, was either ineffectual or even enhanced tumor growth, while the latter significantly inhibited MBT-2 tumor progression.

Both IFN and polyribonucleotides have been effective in initial clinical studies of low grade (18) and occasionally more advanced (22, 23) bladder carcinoma. Larger and more controlled trials will ultimately define the usefulness of polyribonucleotides or IFN in bladder carcinoma. Although either may prove useful as single agents in noninvasive disease, it is probable that a combination of IFN or polyribonucleotides with cytotoxic chemotherapeutic agents will be required for effectiveness in advanced bladder carcinoma. The results of the studies reported here and in in vitro studies, which have defined inhibition of proliferation of established human transitional cell carcinoma cell lines by both naturally produced and recombinant IFN (5, 6, 19), suggest that additional clinical trials are warranted.

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