Effects of Combined Radiotherapy and Immunotherapy With the Use of Pyran Copolymer on Murine Fibrosarcoma

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ABSTRACT—A weakly immunogenic, 3-methylcholanthrene-induced, subcutaneous fibrosarcoma syngeneic to inbred C3H/HeJ mice was used. Pyran copolymer was injected either directly into the tumor, iv, or ip as soon as tumors appeared or when tumors were 8 mm in diameter. One, three, or five doses of pyran copolymer at 10 or 20 mg/kg/dose were injected, with multiple doses being given every other day. Pyran copolymer injected intratumorally once, three times, or five times significantly retarded tumor growth and prolonged the survival times of the hosts. Of the other routes and doses, only pyran copolymer given three times iv significantly retarded tumor growth, but none of these significantly prolonged the survival times of the hosts. Pyran copolymer alone did not induce any complete regression of tumor. Local tumor irradiation with a single exposure to 2,000 rads of X-ray induced complete regressions in some mice, but a higher percentage of tumor cure was observed when tumor irradiation was followed by pyran copolymer treatment.—J Natl Cancer Inst 60: 1477-1481, 1978.

Pyran copolymer, a polyanioncopolymer of divinyl ether and maleic anhydride, was found to induce interferon production in mice and humans (1, 2), to inhibit RNA-dependent DNA polymerase activity of oncogenic virus (3), and to be an effective antitumor agent. In mice bearing Lewis lung carcinoma, B16 melanoma (4), spontaneous mammary tumor, L1210 leukemia (5), or Madison lung carcinoma (6), treatment with pyran copolymer prolonged the survival times of the hosts. When given prophylactically, pyran copolymer rendered mice resistant to subsequent inoculation of tumors (7, 8). When mice bearing LSTRA leukemia or Lewis lung carcinoma were treated with pyran copolymer and BCNU, lasting remissions were observed (9). Pyran copolymer-treated mice exhibited stimulation of the reticuloendothelial system (10), increased antibody formation (11, 12), and increased phagocytosis (13). Snodgrass et al. (14) and Schultz et al. (6) found massive histiocytic infiltration in solid murine tumors after pyran copolymer injection. Macrophages obtained from pyran copolymer-treated mice were found to be cytotoxic to tumor cells in vitro (15). Thus present evidence indicates that pyran copolymer acts through stimulation of the immune system.

We are interested in enhancing the response of tumors to radiotherapy by the use of immunotherapy. In this study, we report the use of combined radiotherapy and pyran copolymer treatment on mice bearing subcutaneous fibrosarcoma (M2). We found that intratumor injections of pyran copolymer significantly retarded tumor growth and prolonged the survival times of the hosts. When pyran copolymer was given after a single exposure to 2,000 rads of X-ray, a higher percentage of tumor cure was observed than that achieved by radiation alone.

MATERIALS AND METHODS

Mice.—Inbred 8- to 10-week-old C3H/HeJ male mice (The Jackson Laboratory, Bar Harbor, Maine) were used. The mice were kept under normal laboratory conditions and provided with Purina Laboratory Chow and tap water ad libitum.

Fibrosarcoma (M2).—M2 is a subcutaneous tumor induced by 3-methylcholanthrene as previously described (16). Immunization of syngeneic mice with irradiated (5,000 R) M2 cells provided a slight degree of protection against a subsequent live M2 tumor challenge. In another experiment, syngeneic mice given 350 rads total body irradiation 24 hours before tumor inoculation were found to be more susceptible to development of tumor as compared to normal controls. These results indicated that M2 tumor is weakly immunogenic and that the immune responses of the hosts do play a role in controlling the growth of this tumor. M2 tumor was maintained subcutaneously in the flanks of C3H/HeJ mice by trocar needle transplantation of pieces of solid tumor. In this study, fifth to seventh generation isotransplants of M2 tumor were used. For a given experiment, tumor cells used were of the same generation.

Single-cell suspension of M2 tumor.—Tumor was dissected out and made into a single-cell suspension by means of enzyme digestion as in (16). For induction of tumors, M2 cells were injected sc into the shaved right hind leg of each mouse. Two perpendicular diameters of each tumor were measured with calipers two to three times a week and the mean diameter was taken as tumor size.

Pyran copolymer (XA146-82-2).—This polymer, with a molecular weight of 18,000, was obtained from Hercules Incorporated (Wilmington, Del.) and solubilized ac-

ABBREVIATION USED: BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea.
justed with saline. Inasmuch as each mouse weighed about 20 g, 0.1- and 0.2-ml doses resulted in doses of approximately 10 and 20 mg/kg, respectively.

Radiotherapy.—Mice were anesthetized lightly with Diabutal (0.025 mg/g) and tumors were irradiated locally as before (16) with a 220-kV X-ray machine. Irradiation was done at 15 mA and 258 rads/minute, with filters of 0.25 mm Cu and 1.0 mm Al and a target-to-surface distance of 27.5 cm.

Statistical analyses.—We used the chi-square test to compare the incidence and Wilcoxon rank sum test to compare the survival times of treated and untreated groups. The difference is considered significant when the P-value is less than or equal to 0.05.

RESULTS

Effect of Route and Dose of Pyran Copolymer Injection on Subcutaneous Fibrosarcoma

Ten groups of mice were inoculated sc with $1 \times 10^4$ M2 tumor cells/mouse on day 0. As soon as tumors appeared (day 12), a single dose of pyran copolymer was injected iv, intratumorally, or ip into each host. For each route of injection, pyran copolymer was given either once, three times, or five times. Multiple doses were given every other day. Pyran copolymer given intratumorally or ip consisted of 0.2 ml/dose, whereas pyran copolymer given iv consisted of 0.1 ml/dose. Results summarized in table 1 show that only pyran copolymer given intratumorally significantly prolonged the survival times of the hosts. Significant inhibition of tumor growth was observed with all doses given intratumorally and three doses given iv.

Effect of Size of Tumor Inoculum and Time of Pyran Copolymer Treatment on Growth of M2 Tumor

In this experiment, 5.5x10^4 M2 cells were injected sc on day 0. Mice were either left untreated or were treated with pyran copolymer five times intratumorally or ip, when the mean tumor size of the group reached 8 mm in diameter. In a separate experiment, we found no statistical difference in the growth rate of the tumors and in the survival times of the hosts with tumors that were untreated and those with tumors treated intratumorally with saline. Therefore, for this and other experiments, untreated tumor-bearing mice were used as controls. Pyran copolymer was given as 0.2-ml doses. Results are plotted in text-figure 1 with results of the corresponding groups from the previous experiment. Mice inoculated with a higher dose of tumor inoculum gave rise to palpable tumors earlier, but once the tumors appeared, the rate of growth of the tumors in both groups was about the same. When pyran copolymer treatment was delayed until the tumors were 8 mm in diameter, inhibition of tumor growth was slight, but not statistically significant.
On the following day, pyran copolymer treatment in 2 of these 3 groups was initiated. Pyran copolymer was given in 0.2-ml doses every other day, five times, either ip or intratumorally. Results are shown in text-figure 2. Radiotherapy alone (X-ray) did not induce any complete tumor regressions. When pyran copolymer treatment was given in combination with tumor irradiation (X-ray plus pyran copolymer), a high percentage of tumors regressed.

This experiment was repeated with $5.5 \times 10^4$ M2 cells, instead of $1 \times 10^4$ M2 cells, for tumor induction. Again, radiotherapy alone did not induce any tumor regression. X-ray plus pyran copolymer treatment given ip five times induced complete but temporary regression in 2 of 6 tumors and complete and lasting regression in 1 of 6 tumors. The remaining tumors in this group regressed slightly but regrew. The same treatment of X-ray plus pyran copolymer given intratumorally induced complete regression in 7 of 8 tumors. Results of this second experiment differed from those of the first in two respects. Firstly, the percentage of tumors cured by treatment of X-ray plus pyran copolymer given ip five times, was much lower in this experiment. Secondly, the time taken for tumors to regress completely after treatment of X-ray plus pyran copolymer given five times intratumorally was much longer in this experiment. These differences may be due to the higher dose of tumor inoculum used in the second experiment, which resulted in a shorter latent period and less immune response to the tumor.

Other schedules of combined tumor irradiation and pyran copolymer treatment were also tested. Results are summarized in table 2. We found that when a lower dose of tumor inoculum was used for tumor induction, tumor cure induced by X-ray combined with pyran copolymer treatments was significantly higher than that induced by X-ray alone. At the higher dose of tumor inoculum, only pyran copolymer given once and five times intratumorally at a 0.2-ml dose in combination with X-ray was significantly better than X-ray alone.

**DISCUSSION**

When mice bearing subcutaneous fibrosarcoma (M2) were treated early with pyran copolymer, tumor growth...
was retarded. Among the three different routes of pyran copolymer injection, iv, ip, and intratumor, inhibition of tumor growth and prolongation of survival times of hosts were most effective with the intratumor route. Pyran copolymer alone did not induce any complete regression of tumor. When pyran copolymer treatment was delayed until tumors reached 8 mm in diameter, very little tumor growth was inhibited. Thus, as with other immunotherapeutic agents, i.e., BCG and Corynebacterium parvum, pyran copolymer is more effective when given early after tumor inoculation.

Chirigos et al. (17) found that daily treatment with low doses of copolymer (25 and 50 mg/kg) resulted in more effective therapy than did a single treatment with a high dose (200 and 400 mg/kg). Therefore, in the present study, low multiple doses of pyran copolymer were used. When the various doses of pyran copolymer were tested for injection, each dose was initially given in 0.2 ml of a 2 mg/ml solution (=20 mg/kg/dose). However, after the first couple of mice were inoculated iv, they appeared sick. Thus the remaining mice in that experiment were inoculated with 0.1 ml pyran copolymer. In a subsequent experiment, we tried giving 0.2 ml of pyran copolymer iv again. This time the mice appeared normal. This phenomenon was not investigated further.

Mohr et al. (9) found that pyran copolymer given after BCNU treatment effectively induced remission of tumor. Our experience with another immunostimulant, C. parvum, also indicated that treatment with immunostimulants is more effective when given immediately after tumor irradiation (16). Therefore, we administered pyran copolymer after irradiation. With a tumor inoculum of 1X10⁴ M2 cells, X-ray and pyran copolymer treatment was always significantly more effective in inducing tumor cure than was radiotherapy alone. With an inoculum of 5.5X10⁴ M2 cells, the difference between X-ray and pyran copolymer treatment and X-ray treatment alone was significant only when pyran copolymer was given once and five times intratumorally in 0.2-ml doses. The finding suggests that the lower dose of tumor inoculum may have elicited a weak immune response in the host and that treatment with pyran copolymer is more effective when such an immune response is originally present.

We do not know the exact mechanism of induction of tumor regression by combined X-ray and pyran copolymer treatment. Work done by other investigators indicated that pyran copolymer treatment in mice stimulated their reticuloendothelial systems (10) and activated macrophages (13). In these respects, pyran copolymer is very similar to C. parvum (18). Milas et al. (19), Suit et al. (20, 21), Moroson et al. (22), and Collins and Song (16) found that administration of C. parvum in combination with tumor irradiation can effectively increase the curability of tumors. It was suggested that irradiation of tumors reduced the tumor load to such an extent that the remaining tumor cells can be eliminated by the cytotoxic effector cells activated by C. parvum treatment. It is likely that pyran copolymer acts in the same manner.

Suit et al. (23) found that the effect of C. parvum combined with radiotherapy depended on tumor immunogenicity; thus the poorly immunogenic tumors responded poorly. The fact that our poorly immunogenic tumor responded well to pyran copolymer plus radiotherapy may indicate that pyran copolymer is a more potent immunostimulant than C. parvum. Furthermore, in our studies with the effect of X-ray plus C. parvum treatment on M2 tumor, we found that C. parvum increased the response to radiotherapy when radiotherapy alone induced some complete regression of tumors (16). However, when radiation alone only induced partial and temporary regressions, C. parvum was ineffective as an adjuvant (Collins AL, Song CW: Unpublished results). In this study, we found that although radiotherapy alone only induced partial and temporary regression of tumors (text-fig. 2), the curability of tumors was increased by X-ray and combined treatment of pyran copolymer.

Merigan and Regelson (2) found that a major side effect of pyran copolymer treatment in man was thrombocytopenia, when a high dose of pyran copolymer was used. We hoped that by reducing the tumor burden with radiotherapy, a small dose of pyran copolymer can be used for controlling tumor growth. No significant cytotoxicity, in terms of decrease in survival time of hosts, was observed in our experiments. Although we did not observe any enhancement of tumor growth after pyran copolymer treatment in this study, we should caution that Kripke and Borsos (24) and Mohr et al. (25) observed, respectively, accelerated development of chemically induced tumors and enhancement of tumor allograft in mice treated with pyran copolymer. Therefore, the use of pyran copolymer in treatment of tumors warrants further study.

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PYRAN COPOLYMER TREATMENT AND RADIOTHERAPY

1481


VOL. 60, NO. 6, JUNE 1978 J NATL CANCER INST