Macrophage Involvement in the Protective Effect of Pyran Copolymer against the Madison Lung Carcinoma (M109)

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SUMMARY

Pyran copolymer (NSC 46015) therapy markedly enhanced host resistance to a murine lung carcinoma (M109) implanted s.c. Multiple dose schedules were not significantly better than single doses at increasing lifespan. Although tumor necrosis was much more extensive in the lesions of pyran-treated mice, pyran copolymer was not directly toxic to M109 cells in vitro. A comparative histopathological study revealed an intense histiocytic reaction in the connective tissue surrounding the primary tumor in mice receiving pyran as compared to 0.9% NaCl solution-treated controls. Macrophages were often associated with necrotic tumor cells. Morphologically activated macrophages were recovered from pyran-treated animals which potently inhibited DNA synthesis of M109 tumor cells in vitro. This response peaked 6 days after drug treatment and was to a large extent specific for necrotic cells. Our results from both in vivo and in vitro studies support the concept that pyran enhances host resistance to neoplasia by mobilization and activation of the reticuloendothelial elements of the host’s defense.

INTRODUCTION

Presently, much attention is being focused on immunotherapy as an adjunct to conventional means of treating neoplastic disease. Pyran (a copolymer of divinyl ether and maleic anhydride) has been shown to suppress the growth of various murine solid tumors (11, 13, 17, 19, 22, 23) and is capable of producing a significant number of “cures” when combined with remission-inducing chemotherapy against the Lewis lung carcinoma and LSTAA murine leukemia (16). Pyran copolymer-induced resistance against neoplasia has been attributed to the effect of pyran on the host’s reticuloendothelial system. A prominent histiocytic (macrophage) infiltrate has been observed at the periphery of the Lewis lung carcinoma in pyran-treated animals as compared to control tumor-bearing mice (22). Furthermore, “activated” macrophages have been recovered from pyran-treated mice which can selectively inhibit the growth of tumor cells in vitro (11, 14, 20, 21). We have shown that macrophage activation by both biological and synthetic agents correlates well with antitumor activity (20). In this paper, we confirm that pyran enhances resistance of BALB/c mice to another transplanted, syngeneic lung carcinoma (M109), and we present further in vivo and in vitro evidence which implicates activated macrophages as effectors of this pyran-induced antitumor resistance.

MATERIALS AND METHODS

Mice. Male BALB/c mice, 6 to 8 weeks old, were furnished by the Mammalian Genetics and Animal Production Section, Drug Research and Development, NIH, Bethesda, Md. The animals were housed in plastic cages and given Purina laboratory chow and tap water ad libitum. All animals weighed at least 23 g before they were used for experimentation.

Tumor Testing. The Madison lung carcinoma, a transplantable line derived from a spontaneous neoplasm in a BALB/c mouse, was kindly supplied by Dr. Ruth I. Geran, Drug Research and Development, National Cancer Institute, NIH, in its 110th passage generation. The line was subsequently adapted to tissue culture. For adjuvant studies, 5 x 10⁶ viable M109 cells, suspended in serum-free Roswell Park Memorial Institute Medium 1640, were injected s.c. into the right inguinal region of each mouse. Deaths of mice were recorded daily, and median survival times were calculated. The percentage increase in life-span of test groups (T) over control groups (C) inoculated with tumor alone was calculated by (T/C - 1) x 100. The mean survival of pyran-treated groups in comparison to those of groups receiving 0.9% NaCl solution were evaluated statistically by Student’s t test.

Pyran Copolymer. Pyran-2-succinic anhydride-4,5-dicarboxyterahydroxy-6-methyl anhydride polymer (NSC 46015) was kindly supplied by Dr. David Breslow of Hercules Research Center, Wilmington, Del. Pyran was dissolved in 0.9% NaCl solution, adjusted to pH 7.0 by the addition of 1 N NaOH, and given i.p. at 1% body weight.

Target Cells. Tissue culture strains of M109 and B16 melanoma cells were initiated from in vivo transplant lines serially carried in this laboratory. These cells were maintained in RPMI-FCS.² M109 cells were used during passages 12 to 20, and B16 melanoma was used during passages 3 and 4. At intervals, the oncogenicity of the cell lines was proven by observing 100% tumor incidence in syngeneic mice inoculated with 1 x 10⁶ cultured cells. Two “normal” contact-inhibited cell lines, NCTC 1469 and 3T3-Swiss albino, were obtained from the American Type Culture Col-

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lection, Rockville, Md. These cells were similarly adapted for growth in RPMI-FCS.

**Toxicity Assay.** M109 tissue culture cells were dispersed with 0.25% trypsin and 0.02% EDTA, and viable cells were determined by trypsin blue exclusion. Viable cells (1.0 x 10^6) were plated in 25-sq cm tissue culture flasks and incubated at 37° for 24 hr. Growth medium was then removed, and new RPMI-FCS containing pyran copolymer in concentrations from 10 to 10,000 µg/ml was added. The cells were incubated for an additional 24 hr and were trypsinized, and viable cells were determined by trypsin blue exclusion. Three flasks were used for each drug concentration, and the mean cell count and S.E. were calculated.

**Peritoneal Macrophages.** Peritoneal exudates were harvested from BALB/c mice by i.p. injection of 5 ml of serum-free Roswell Park Memorial Institute Medium 1640 containing 2 units of heparin per ml. Within 10 min after injection, the mice were sacrificed, and the peritoneal exudate was collected by paracentesis. Exudates from 10 mice in each group were pooled, washed once in 30 ml RPMI-FCS, and purified by adherence as previously described (21). Representative preparations of purified peritoneal adherent cells were stained with Giemsa stain; >95% of the cells seen had morphological characteristics of macrophages. Macrophage suspensions were kept in an ice bath prior to use to prevent adherence.

**Growth Inhibition Test.** Macrophage-mediated cytostasis of the target cells was measured by inhibition of DNA synthesis. Target cells were trypsinized from exponentially growing cultures and resuspended at 5.0 x 10^4 cells/ml RPMI-FCS, and 2 ml were placed in 30-mm tissue culture dishes. Purified peritoneal macrophages were adjusted to 1.0 x 10^5 cells/ml RPMI-FCS, and 1 ml was added to the target cell cultures; the effector:target cell ratio was, therefore, 10:1. The DNA synthesis of the target cells was assessed after 20 hr of incubation at 37°. Triplicate cultures of each set of dishes as well as cultures consisting of tumor cells alone and macrophages alone were pulsed with 1.0 µCi of tritiated thymidine (specific activity, 10 Ci/m mole; New England Nuclear, Boston, Mass.; NET-355) for 2 hr at 37°. At the end of the incubation, the cells were detached with 0.25% trypsin for 20 min, centrifuged at 600 x g for 10 min, and resuspended in 0.5 ml Dulbecco’s phosphate-buffered saline. Four ml of chilled 10% trichloroacetic acid were added to each tube, and precipitation was allowed to go for 30 min at 4°. The resultant precipitate was collected on glass-fiber paper (Millipore Corp., Bedford, Mass.) and washed with cold 10% trichloroacetic acid (approximately 200 ml/sample). The filters were then air dried and assayed for radioactivity in a Packard Tri-Carb scintillation counter using Aquasol solubilizer (New England Nuclear). The percentage specific inhibition of DNA synthesis was calculated by the formula:

\[
\text{% specific inhibition} = \left( \frac{\text{cpm}_N - \text{cpm}_E}{\text{cpm}_N} \right) \times 100
\]

where cpmN = mean cpm in cultures containing effector cells from normal control mice and cpmE = mean cpm in cultures containing test effector cells. *p* values were calculated by Student’s *t* test.

**Histopathology.** Whole tumors were excised at different time intervals after tumor inoculation and fixed in 10% neutral formalin. These tissues were sectioned and routinely stained with hematoxylin and eosin.

**RESULTS**

**Antitumor Activity of Pyran in Vivo.** Pyran copolymer at 25 mg/kg significantly prolonged the median survival time in all treated groups of mice bearing the Madison lung carcinoma (Chart 1). A minimum of a 50% increase in life-span over 0.9% NaCl solution-treated controls was achieved regardless of the time of treatment initiation or whether a single or multiple treatment regimen was used. A single treatment late in the course of disease (Day 14) was the least effective therapeutic regimen at increasing life-span (p < 0.02), whereas all other schedules were highly significant (*p* < 0.001). Although multiple treatments with pyran were slightly more effective in increasing life-span than single treatments, this difference was not statistically significant. Since pyran copolymer therapy at Day 7 consistently produced the best antitumor response of the single-treatment groups, this regimen was chosen to determine the mechanism of pyran’s antitumor activity.

**In Vitro Toxicity of Pyran.** At concentrations of up to 1 mg/ml, pyran copolymer did not significantly affect cellular growth or cell viability of M109 cells (Table 1). The 10 mg/ml concentration resulted in significant cytostasis, although cell viability was not diminished.

**Local Tumor and Host Response.** A comparative histopathological study was performed on the primary M109 tumor in BALB/c mice that received pyran or 0.9% NaCl solution treatment on Day 7. The results show that the s.c. implanted tumors of all animals that received pyran were clearly separated from the connective tissue of the host. In contrast, the demarcation of the tumor mass from the connective tissue was less distinctive in animals that received 0.9% NaCl solution. As early as 2 days after pyran inoculation (Day 9), the slightly compressed tumor showed a moderate infiltration with histiocytes. Isolated groups of tumor cells at the tumor periphery showed signs of early degeneration and were surrounded by histiocytes (Fig. 1). Small areas of central necrosis developed in the tumor at this time. At Day 9 in the 0.9% NaCl solution-treated group, there was a moderate infiltration of the dermis and subcutis with mononuclear cells (monocytes and histiocytes) as well as many polymorphonuclear leukocytes; the latter were seen in and around small venules in this area. At Day 13, the tumor in pyran-treated mice appeared to be completely surrounded by a broad band of connective tissue which was heavily infiltrated with histiocytes. A large amount of histiocytes also infiltrated toward the center of the primary tumor mass (Fig. 2); this resulted, finally, in a separation with multiple, small nodular tumor units at Day 27 (Fig. 3). Histiocytes in the periphery of these tumor nodules were closely associated with necrobiotic carcinoma cells (Fig. 4). In the central areas of the tumor as well as in the periphery, many areas of coagulative necrosis appeared at late intervals (Days 20 and 27). Large parts of the tumor were replaced by granulation tissue heavily infiltrated with histiocytes and...
In vitro toxicity of pyran copolymer for M109 carcinoma cells.

Table 1

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<tr>
<th>Dose of pyran</th>
<th>Growth inhibition (%)</th>
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<tr>
<td>Nil</td>
<td>0</td>
<td>90</td>
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<tr>
<td>500 µg/ml</td>
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<td>1 mg/ml</td>
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<td>90</td>
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<td>10 mg/ml</td>
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a Pyran copolymer was added to exponentially growing M109 cultures as described in text. The cultures were terminated after 24 hr of incubation, and the number of total M109 cells per flask was determined.

b Percentage viability of M109 cells was determined by trypan blue exclusion.

Effect of Pyran Copolymer on in Vitro Morphology of Peritoneal Macrophages. Peritoneal macrophages from mice treated i.p. with pyran copolymer were large vacuolated cells with a marked propensity to stretch out on glass as compared to normal macrophages. Four hr after initiation of cultures, macrophages from pyran-treated mice were well spread out, whereas most of the normal macrophages remained rounded up (Figs. 7 and 8). Macrophages from treated mice also possessed numerous phase-dense granules and phase-lucent vacuoles. These morphologically activated macrophages could be recovered from mice 4 days after pyran administration and persisted for at least 20 days.

Macrophage-mediated Cytostasis of Target Cells. Peritoneal macrophages were harvested at various times after pyran inoculation from normal and tumor-bearing mice and tested in vitro for their ability to inhibit M109 cell DNA synthesis (Chart 2). Macrophages from control nontreated M109 tumor-bearing mice specifically inhibited M109 cell DNA synthesis (32%) when taken from mice 14 days after tumor inoculation. No significant inhibitory activity was observed prior to or after this period. Pyran treatment given i.p. to normal and M109 tumor-bearing mice markedly enhanced macrophage inhibition of M109 cell DNA synthesis (32%) when taken from mice 14 days after tumor inoculation. No significant inhibitory activity was observed prior to or after this period. Pyran treatment given i.p. to normal and M109 tumor-bearing mice markedly enhanced macrophage inhibition of M109 cellular DNA synthesis. Pyran potentiated macrophage-mediated cytostasis by Day 12 (5 days after treatment) and reached peak values by Day 13 (6 days after treatment). Activity was maintained at a high level for 13 days after treatment. In both normal and tumor-bearing mice, pyran treatment resulted in an earlier, higher, and longer-lasting macrophage-mediated cytostasis. This response was consistently higher in tumor-bearing mice, indicating that pyran activation of the “armed” (specifically cytostatic) macrophages appeared to work synergistically.

To determine the tumor specificity of this response, the cytostatic effect of pyran-activated macrophages was also...
tested against the B16 melanoma, NCTC clone 1469, and 3T3-Swiss albino cell cultures. Macrophages were harvested 7 days after pyran treatment of normal mice and used as effector cells. Cytostatic activity was greatest against the B16 cell line (64.9% specific inhibition), but activity was also definitely present against the 3T3 cells (38.3%). In contrast, stimulatory effects on the growth of the normal NCTC 1469 epithelial line were observed (−26%).

DISCUSSION

Pyran copolymer (NSC 46015) was used in single or multiple treatment regimens with treatment being initiated at different time intervals to test the ability of the drug to alter the growth of a poorly differentiated lung carcinoma (M109). The observations reported here support and extend the finding of Woodman and Gang (23), that pyran therapy is beneficial against the M109 tumor (64 to 125% increase in life-span), and provide general indications concerning the use of pyran. Dosage in this study was maintained at 25 mg/kg/day, although preliminary results suggest that pyran is active over a wide range of doses from 1 to 100 mg/kg/day. Multiple treatments were found not to be statistically better than single treatments. Prolonged survival was accomplished even when treatment was delayed as late as 14 days after tumor inoculation.

Pyran copolymer was not toxic to M109 cells in vitro, and a concentration of 10 mg/ml was required to inhibit 50% of cell growth. Nevertheless, pyran was effective in vivo at 25 mg/kg on treatment schedules that provided cumulative doses of approximately 500 to 4500 μg/mouse. The results reported here are in agreement with those of Kaplan et al. (14), that direct tumor cell cytotoxicity is not a major mechanism for the antitumor activity of pyran. Therefore, the protective effect exerted by pyran was considered to be due to modulation of the host's antitumor defense mechanisms.

Recent evidence strongly implicates macrophages as major effectors of the antitumor resistance induced by both synthetic and biological adjuvants. Peritoneal macrophages taken from mice pretreated with such biological stimulants as Corynebacterium parvum (9, 18); Mycobacterium bovis, strain Bacillus Calmette-Guérin (4, 8, 12); and Toxoplasma gondii (15) are activated to inhibit the growth of tumor cells in vitro. Similar responses have been reported for synthetic agents such as pyran copolymer (11, 14, 20, 21) and double stranded RNA (1). This cytotoxicity expressed by activated macrophages is purported to be specific for neoplastic as opposed to normal cells but is immunologically nonspecific with regard to different tumor cells (12, 14). We report here that macrophages recovered from pyran-treated mice effectively inhibit DNA synthesis of tumor cells in vitro; this activity was found to peak at 6 days after drug administration and was largely tumor specific. Activation of macrophages by pyran copolymer does not appear to be dependent on T-cell factors, since cytotoxic macrophages have been recovered from irradiated, bone-marrow reconstituted mice lacking T-lymphocytes (14) and from athymic, nude mice (21). Moreover, preliminary results in our laboratory suggest that pyran can directly render purified peritoneal macrophages cytostatic for tumor cells in vitro.

It has been reported that macrophages may also be armed (made specifically cytotoxic) following interaction with specifically sensitized T-lymphocytes (7). We recently reported that adjuvant-induced potentiation of armed macrophage cytotoxicity correlated well with the ability of agents to enhance antitumor resistance (20). Of the chemicals tested, pyran was found to be the most effective adjuvant. Similarly, we show here that pyran worked synergistically to augment specific macrophage reactivity in a syngeneic tumor system.

The presence of large numbers of histiocytes in several untreated animal tumors has been associated with tumor rejection and the lessened likelihood of metastasis (2, 5, 6). Black (3) noted that syncytial and sinusoidal histiocytosis in the lymphatic tissue draining human mammary carcinomas could be correlated with a prognostically favorable course. Hanna et al. (10) showed that induction of a histiocytic granuloma with Bacillus Calmette-Guérin at the tumor site was required for the rejection of a transplantable hepatocarcinoma in guinea pigs. Similarly, i.p. treatment with pyran copolymer provoked a marked histiocytic reaction around the s.c. transplanted Lewis lung carcinoma and its metastases in C57BL/6 mice (22). We observed that pyran therapy against the M109 lung carcinoma involved an analogous mode of action; the tumor in pyran-treated mice was compressed by a broad band of connective tissue heavily infiltrated with histiocytes and macrophages as compared to 0.9% NaCl solution-treated controls, halting the tumor's infiltration and separating the tumor into multiple, small nodular units. Necrosis was shown to be more extensive in the lesions of pyran-treated mice, and histiocytes were often associated with degenerate-appearing tumor cells.

Thus, pyran copolymer induces host resistance to the growth of the Madison lung carcinoma through hyperplasia and activation of the reticuloendothelial system. Pyran therapy resulted in: (a) enhanced macrophage inhibition of tumor cell DNA synthesis, (b) heavy accumulation and infiltration of histiocytes and macrophages at the tumor site, and (c) the association of histiocytes with degenerate-ap-
peaning tumor cells. The cure rate attained when pyran was combined with remission-inducing chemotherapy (16) and its effect on modulating host immunological factors, even in an advanced tumor system, strongly support the potential use of pyran copolymer as an adjuvant to other conventional tumor treatment modalities.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Local tumor with early degeneration of tumor cells (arrows) isolated by histiocytic connective tissue, 2 days after pyran treatment. × 250.
Fig. 2. Connective tissue heavily infiltrated with histiocytes growing from the periphery toward the center of the tumor mass, 7 days after pyran treatment. × 250.
Fig. 3. Segregation of tumor mass into smaller tumor nodules surrounded by histiocytes and macrophages in connective tissue, 21 days after pyran treatment. × 125.
Fig. 4. Histiocytes are in close association with necrobiotic tumor cells (arrows) in the periphery of the tumor nodule. × 500.
Fig. 5. Macrophages with typical foamy appearance (arrow, a) and vacuoles (arrow, b) in the abundant cytoplasm, 21 days after pyran treatment. Oil immersion, × 625.

Fig. 6. Tumor surrounded by connective tissue without any host reaction. Many mitotic figures are in the poorly differentiated carcinoma, 21 days after 0.9% NaCl solution treatment. × 250.

Fig. 7. Normal BALB/c mouse peritoneal macrophages after 4-hr culture in plastic dishes. Phase contrast, × 250.

Fig. 8. Normal BALB/c mouse peritoneal macrophages 6 days post-pyran copolymer treatment (4 hr in culture). Note cells stretching out with numerous phase-dense granules and phase-lucent vacuoles. Phase contrast, × 250.
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