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# Enhancement of a Tumor Allograft in BALB/c × DBA/2 $F_1$ Mice by Pyran Copolymer

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## SUMMARY

Pyran copolymer (NSC 46015) was evaluated with respect to its effect on the rejection of a murine leukemic allograft by BALB/c × DBA/2 $F_1$ ($CD2F_1$) mice. Significant prolongation of allograft survival with production of progressively growing lethal tumors was found following pyran administration. This phenomenon occurred at nontoxic doses of the drug and appeared to be closely related to the timing of pyran injection. Nonspecifically stimulated lymphocyte blast transformation by concanavalin A was not impaired by pyran when lymphocytes were exposed *in vitro* to the drug. The mechanism of tumor allograft enhancement remains obscure but may be related to allograft size at the time of pyran administration.

## INTRODUCTION

The use of chemical and biological agents capable of modifying the immunological response of the host, particularly against cancer cells, has been an area of intense investigation. Pyran (NSC 46015), the polyanionic copolymer of divinyl ether and maleic anhydride, has been shown to induce interferon (23), inhibit the activity of the DNA polymerase of avian myeloblastosis virus (29), and protect against nononcogenic (4, 8, 24) and oncogenic (6, 15, 16) virus infection. In addition, pyran has been demonstrated to be a potent activator of macrophages (17, 28, 33) and a stimulator of antibody formation (2). These effects on the immune system of the host probably account for the beneficial responses achieved in experimental murine tumor systems using pyran therapy alone (13, 18, 27, 34) or as an adjuvant to conventional anticancer therapy (26).

In contrast to these studies in which pyran copolymer appeared to offer promise as an antitumor agent, several investigators have found enhanced tumorigenesis in conjunction with its administration (11, 20, 35). However, the mechanism(s) by which pyran treatment augmented viral or chemical-induced oncogenesis has remained unclear. In this report, the unexpected finding that pyran therapy also enhanced the growth and lethality of an *H-2*-incompatible tumor graft is described and the relation of this phenomenon to the antigenic mass of the allograft is noted.

## MATERIALS AND METHODS

**Tumor.** The MBL-2 murine leukemia, used as an allograft in these studies, was originally induced in young C57BL/6J ($H\text{-}2^b$) mice by the Moloney leukemia virus. This leukemia has been passed in the ascites form in this laboratory for over 200 generations in syngeneic C57BL/6J mice. Viability was determined by trypan blue exclusion, and the desired number of cells was injected s.c. in the inguinal region in 0.2 ml of Eagle's minimal essential medium. Tumor size was monitored at 2- to 3-day intervals by manual palpation and metal caliper measurement and was reported as the average of 2 perpendicular diameters. To avoid confusion with scar tissue, tumors were not considered significant unless they were larger than 3.0 mm in diameter, and animals with tumors larger than 15 mm in diameter after 35 days were considered to have progressive disease, eventually having a 100% mortality rate.

**Mice.** Adult male BALB/c × DBA/2 $F_1$ mice (hereafter called $CD2F_1$) ($H\text{-}2^d$) mice were used as recipients of the MBL-2 allograft. All mice used were 6 to 8 weeks old and weighed approximately 25 g. Mice were housed in plastic cages with filter bonnets and fed Purina laboratory chow and water *ad libitum*. Animals were supplied by the Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Md.

**Drugs.** Pyran copolymer (NSC 46015) was supplied by Hercules, Inc., Wilmington, Del. All preparations were made fresh daily in 0.9% NaCl solution, and the pH was adjusted to 7.0 with 1.0 N sodium hydroxide. All injections were i.p. in a volume equal to 1% body weight. The 0.9% NaCl solution was injected into control animals.

**Preparation of Spleen Cells.** Spleens were removed from normal or pyran-treated $CD2F_1$ mice following their sacrifice by cervical dislocation. They were washed 3 times in 0.9% NaCl solution and then minced to a fine gel. The splenic gel was resuspended in 0.9% NaCl solution, and particulate matter was removed by filtration through 4 layers of gauze. The filtrate was centrifuged at 1000 × *g* for 10 min and, to lyse the erythrocytes, the cellular pellet was suspended in sterile distilled water for 15 to 20 sec, followed by rapid equilibration to 0.9% NaCl solution by the addition of 3.5% NaCl solution. The cell suspension was then centrifuged at 1000 × *g* for 10 min and the supernatant was discarded. The purified lymphocytes were resuspended to a concentration of $1.0 \times 10^6$/ml in Roswell Park Memorial Institute Medium 1640 containing 10% fetal calf serum, 100 units penicillin per ml, and 100 μg streptomycin per ml after counting with trypan blue to determine viability.

**Lymphoblastogenesis with the use of Con A.**[1] Incubation

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[1] The abbreviation used is: Con A, concanavalin A.

for 48 hr at 37° in 5% $CO_2$ was performed with sterile capped 12- x 75-mm plastic tubes containing 1.0 × $10^6$ lymphocytes. Lymphoblastogenesis was stimulated by the addition of 2.0 μg of Con A (Calbiochem, San Diego, Calif.) in 50 μl sterile 0.9% NaCl solution to the incubation tube at time zero. Pyran was made in sterile 0.9% NaCl solution, pH 7.4, and added in 50-μl volumes as well. A pulse of 2.0 μCi of [$^3$H]thymidine was added to the incubation tube 4 hr prior to termination of the experiment. The incubation was stopped by washing the labeled cells once in 0.9% NaCl solution and by precipitation of acid-insoluble product by ice-cold 10% trichloroacetic acid containing 0.01% ATP. The precipitate was collected by filtration over a Whatman GF/c glass membrane, washed with several volumes of cold 10% trichloroacetic acid, air dried, and counted in a liquid scintillation counter. Background counts obtained from the extraction of pulsed incubation media have been subtracted from the reported cpm.

## RESULTS

The response of $CD2F_1$ (*H-2*$^d$) mice to the MBL-2 (*H-2*$^b$) allograft was as anticipated (Chart 1). Within 1 week of inoculation, easily palpable tumor was present in all animals, and the allograft slowly decreased in size so that 50% regression occurred by Day 17 with complete rejection by Day 24. Pretreatment with pyran 7 days prior to tumor inoculation shifted the typical rejection profile slightly toward a shorter time course, with the average tumor size consistently smaller than that of controls. In contrast, pyran injection 6 days after allografting markedly enhanced the survival of the allograft. Approximately 90% of the animals developed progressively growing tumors that had an average diameter of 19.2 mm on Day 34 and that eventually caused their demise.

To determine whether this unexpected observation was dose dependent, the dosage of pyran injected on Day 6 after allografting was varied (Table 1). Allograft enhancement disappeared at doses below 2.5 mg/kg and progressively increased with dosages up to 20 mg/kg. Little further increase was seen above 20 mg/kg and, at doses greater than 100 mg/kg, the animals suffered progressive weight loss and toxic early deaths. Therefore, prolonged graft survival was clearly dependent on pyran dosage but occurred at doses that were clinically nontoxic and of previously demonstrated therapeutic benefit in syngeneic tumor systems (26).

To exclude the possibility that pyran might have a generalized toxicity directed toward splenic lymphocytes that might result in allograft enhancement, nonspecific *in vitro* blast transformation by concanavalin A was used to test the response of splenic lymphocytes exposed *in vitro* to pyran. Lymphoblastogenesis was inhibited only at an *in vitro* pyran concentration of 500 μg/ml (Table 2). To achieve this drug level in a mouse would require a lethal dose of approximately 350 mg/kg, given that roughly 70% of the murine body weight equals total body water (1). Therefore, it was unlikely that a generalized lymphocyte toxicity occurred at the doses of pyran used to produce allograft enhancement.

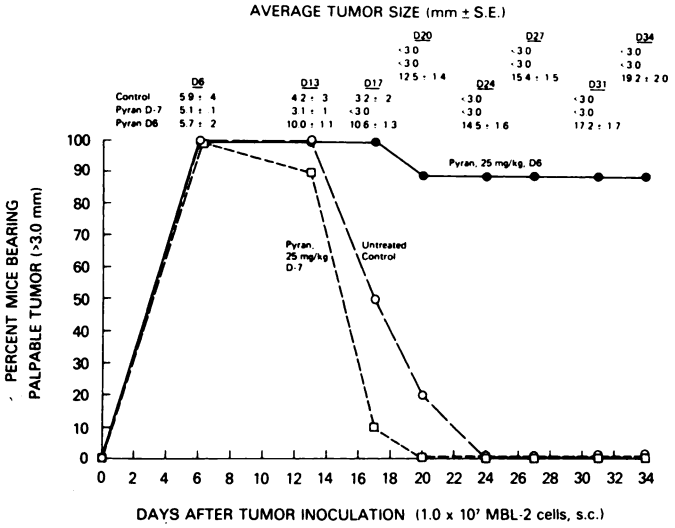


Chart 1. Time course of MBL-2 allograft rejection in normal and pyran-treated $CD2F_1$ mice. Mice were inoculated s.c. on Day 0 with 1.0 × $10^7$ MBL-2 cells in 0.2 ml Eagle's minimal essential medium. The percentage of mice bearing tumors at least 3.0 mm in diameter is indicated for specific observation days, and the average tumor size on that day is shown directly above. Pyran was injected at a dose of 25 mg/kg i.p. in an injection volume equal to 1% body weight. There were 10 animals/group.

Table 1

*Effect of pyran dosage on MBL-2 allograft enhancement*

Normal $CD2F_1$ mice were inoculated s.c. with 1.0 × $10^7$ MBL-2 cells on Day 0.

| Pyran dosage, i.p., Day 6 (mg/kg) | Tumor-bearing mice[a]/total no. of mice | Av. tumor size on Day 25 (mm) |
|---|---|---|
| 0.9% NaCl solution control | 0/10 | <3.0 |
| 0.1 | 0/10 | <3.0 |
| 1.0 | 0/10 | <3.0 |
| 2.5 | 2/10 | 12.5 ± 1.5[b] |
| 5.0 | 3/10 | 5.7 ± 1.8 |
| 10.0 | 4/10 | 7.2 ± 2.4 |
| 20.0 | 7/10 | 7.6 ± 1.1 |
| 40.0 | 6/10 | 8.0 ± 1.7 |
| 100.0 | 8/10 | 10.0 ± 1.4 |

[a] Bearing palpable tumor (>3.0 mm) 25 days after tumor inoculation.

[b] Mean ± S.E.

Pyran copolymer was given at a time when the allograft was near maximal size. To determine whether the time of pyran administration was critical for the enhancement phenomenon to occur, pyran was given at different time intervals up to 14 days after MBL-2 implantation (Table 3). Pyran administration on Day 6 after tumor inoculation impaired allograft rejection most effectively, but significant enhancement was also observed if pyran was given on Day 4, 8, or 10. Pretreatment with pyran prevented the 20% incidence of progressive tumor in control animals that occurred in this experiment. Pyran did not seem to produce significant enhancement if given early or late in the course of allograft rejection (compare with Chart 1) and, therefore, it appeared that allograft size might be related to this observation.

To assess further whether allograft size at the time of pyran injection could affect the enhancement phenomenon,

Table 2
*Effect of pyran on in vitro lymphoblastogenesis by Con A*

| Pyran concentration[a] (μg/ml) | cpm[b] × $10^{-3}$ splenic lymphocytes[c] | |
|---|---|---|
| | −Con A | +Con A[d] |
| Control | 4.3 ± 0.3 | 295.0 ± 23.3 |
| 15.6 | 8.3 ± 0.7 | 316.4 ± 14.4 |
| 31.2 | 5.1 ± 0.8 | 266.7 ± 10.1 |
| 62.5 | 8.5 ± 0.7 | 239.9 ± 11.6 |
| 125 | 4.4 ± 0.2 | 258.2 ± 23.0 |
| 250 | 1.6 ± 0.1 | 202.4 ± 10.6 |
| 500 | 3.1 ± 0.1 | 53.4 ± 2.2 |

[a] In 1.0 ml incubation medium.
[b] Results, cpm (mean ± S.E.) of quintuplicate samples, rounded to the nearest 100.
[c] One × $10^6$ splenic lymphocytes from normal $CD2F_1$ mice per incubation tube in 1.0 ml Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum, 100 units penicillin per ml, and 100 μg streptomycin per ml.
[d] Con A, 2.0 μg/incubation tube.

Table 3
*Effect of time of pyran therapy on MBL-2 allograft enhancement*
Normal $CD2F_1$ mice were inoculated s.c. with 1.0 × $10^7$ MBL-2 cells on Day 0.

| Day of pyran therapy, 25 mg/kg, i.p. | Animals with progressive tumor[a]/total no. of animals | Av. tumor size on Day 45 (mm) |
|---|---|---|
| None | 2/10 | 22.5 ± 0.5[b] |
| −7 | 0/10 | <3.0 |
| −5 | 0/10 | <3.0 |
| −1 | 0/10 | <3.0 |
| 0 | 1/10 | 26.0 ± 0 |
| 2 | 2/10 | 26.0 ± 3.0 |
| 4 | 5/10 | 23.6 ± 1.8 |
| 6 | 7/10 | 20.7 ± 2.4 |
| 8 | 6/10 | 23.8 ± 2.9 |
| 10 | 5/10 | 20.2 ± 1.5 |
| 12 | 4/10 | 21.2 ± 2.6 |
| 14 | 1/10 | 25.0 ± 0 |

[a] Mice bearing progressively growing tumor allografts (>15.0 mm) 45 days after inoculation.
[b] Mean ± S.E.

the inoculum of MBL-2 allograft was varied, keeping the dose and time of pyran injection constant (Table 4). The size of palpable tumors on Day 6 progressively increased with increasing tumor inoculum, but there was little difference between pyran-treated and 0.9% NaCl solution control groups in tumor incidence. However, the pyran-treated groups developed progressively growing allografts with a much greater frequency than the control groups, and this effect was most pronounced at the 1.0 × $10^6$ or $10^7$ tumor cell inoculum levels, tapering off at higher allograft doses. Hence, it appeared that the allograft size at the time of pyran injection was related to the enhancement process.

## DISCUSSION

A dose-related phenomenon of enhanced tumor allograft survival with increased incidence of progressively growing tumors following pyran copolymer administration has been described. However, the mechanism(s) by which this occurs remains unclear. Allograft rejection is dependent upon the T-lymphocyte (5, 21, 22), although there is now increasing evidence that macrophages may become the actual effector cells after specific arming by T-cell-derived products (10, 19). Although by no means conclusive, the failure to demonstrate inhibition of Con A-induced lymphoblastogenesis with pyran (Table 2) suggests that at least a generalized lymphocyte toxicity probably does not occur with pyran administration at the usual dose of 25 mg/kg. Furthermore, we have found that splenic lymphocytes taken from mice 48 hr after treatment with that dose of pyran respond as well as do controls to Con A (unpublished observations). However, since the cell population responding to Con A is not necessarily the specific population involved with rejection of the MBL-2 allograft, antigen-specific cellular-cytotoxicity experiments would be necessary to entirely clarify this point.

In the experiments reported here, the antigenic mass of tumor allograft present at the time of pyran injection appeared to be related to the frequency of allograft enhancement, and similar observations have been noted in other syngeneic and allogeneic systems (3, 37). The mechanism of this relationship is unclear, although it has been shown that certain neoplasms can produce factors that subvert the normal immune response (12, 30, 36). Hence, the host immune responses and the growing alloantigenic mass are in a state of constant flux, in which rapid allograft growth occurs initially in spite of intact host immunity, followed by allograft rejection by the eventually dominating *H-2*-incompatibility response. It is possible that during this interaction, there occurs a period during which the process is very sensitive to pyran administration, with the resulting outcome in favor of the allograft. This would help explain the data in Table 3 which indicate that pyran treatment had no effect if given before allografting and little effect if given early or late in the course of allograft rejection. It also agrees with the data in Table 4, in that the pyran-sensitive point may not have been reached by Day 6 in the lower tumor inoculum groups, whereas it is already passed in the high tumor inoculum groups. The low progressor incidence at high tumor inoculum might be at least partially explained in this way. Therefore, the state of the dynamic interaction between the allograft and the immune system may be the key factor determining allograft enhancement and not really allograft size, which is only an indirect measure of this interaction. Again, more specific immune testing is necessary to explain the phenomenon further.

The exact nature of pyran's effect in this system when given at the appropriate time is unknown. Hyperimmune antibody can inhibit cellular immunity in allogeneic systems (7, 14, 25), and certain populations of lymphocytes can suppress normal immune responses in both syngeneic (31, 32) and allogeneic (9) systems, the latter apparently being a bursa-dependent lymphocyte population. Since pyran can stimulate the antibody-producing or B-cell compartment of immunity (2), this activity may account for its enhancing effect.

The normal allograft immune response is certainly quantitatively and probably qualitatively different from the response of a syngeneic host to its tumor. It remains unclear

Table 4
*Allograft enhancement by pyran as related to varying allograft dose*

| Tumor cell inoculum[a] | Av. tumor size on Day 6 (mm) | Tumor incidence[b] | | Animals with progressive tumors[c] | |
|---|---|---|---|---|---|
| | | Control[d] | Pyran treated[e] | Control[d] | Pyran treated[e] |
| $1.0 \times 10^3$ | <3.0 | 4/10 | 7/10 | 2/10 | 0/10 |
| $1.0 \times 10^4$ | <3.0 | 6/10 | 7/10 | 2/10 | 3/10 |
| $1.0 \times 10^5$ | <3.0 | 8/10 | 10/10 | 0/10 | 3/10 |
| $1.0 \times 10^6$ | 3.8 ± 0.2[f] | 10/10 | 10/10 | 0/10 | 6/10 |
| $1.0 \times 10^7$ | 6.2 ± 0.3 | 10/10 | 10/10 | 0/10 | 7/10 |
| $1.0 \times 10^8$ | 8.8 ± 0.2 | 10/10 | 10/10 | 0/10 | 3/10 |
| $1.0 \times 10^9$ | 9.5 ± 0.3 | 10/10 | 10/10 | 0/10 | 2/10 |

[a] Number of MBL-2 tumor cells injected s.c. on Day 0.
[b] Maximal number of animals developing palpable tumor (>3.0 mm)/total number of animals in group.
[c] Animals developing progressively growing tumors (>15.0 mm) by Day 35/total number of animals in group.
[d] Given injections of 0.9% NaCl solution 0.25 ml, i.p., Day 6.
[e] Pyran copolymer, 25 mg/kg, i.p., Day 6. ., Day 6.
[f] Mean ± S.E.

why pyran can be beneficial in syngeneic tumor systems and yet can enhance the growth of a tumor allograft as reported here. Since pyran may be useful in transplantation and oncological medicine, the need for further immunological testing of this drug is indicated.

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