Specific Potentiation of L1210 Vaccine by Pyran Copolymer

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SUMMARY

Pyran copolymer (NSC 46015) was found to potentiate strongly the immune response of C57BL/6J × DBA/2 F1, mice to 10^4 live L1210 tumor cells following suboptimal vaccination with 10^5 radiation-inactivated L1210 cells. Optimal immunity to challenge was produced by concomitant i.p. administration of pyran and L1210 vaccine, and activity was dependent upon both pyran and vaccine dosages. In addition, this immunopotentiation seemed to be related to the intrinsic viscosity of different pyran preparations tested, although all the pyran compounds had significant activity. Furthermore, the increased immunity to subsequent live tumor challenge appeared to be specific for the vaccinating cell type.

INTRODUCTION

Agents capable of specifically modifying host immune responses in a beneficial manner against a syngeneic neoplasm are being sought and studied in that they may provide an additional weapon in the prevention and treatment of cancer. In this respect, investigations with pyran copolymer (NSC 46015), the polyanionic copolymer of maleic anhydride and divinyl ether, appear promising, since pyran has been shown to: (a) inhibit the activity of the DNA polymerase of avian myeloblastosis virus (19); (b) induce interferon (13); (c) protect against nononcogenic virus infection (3, 6, 14); (d) prevent viral-induced oncogenesis (5, 9, 10); (e) stimulate antibody production (1); (f) enhance macrophage activity (11, 18, 21); and (g) exert a beneficial therapeutic effect by itself in various experimental murine tumor systems (7, 12, 17, 22). Recently, it has been reported that pyran can act as a valuable adjuvant to remission-inducing chemotherapy of a murine leukemia and solid tumor (16). Further evidence that pyran can be useful as an adjuvant is now presented.

To demonstrate more clearly this adjuvant capability, pyran was combined with a vaccine prepared by radiation of L1210 ascites cells and used in a suboptimal manner. The poor immunity to subsequent L1210 challenge was strongly augmented by the addition of pyran as an adjuvant to the vaccine. Furthermore, this potentiation of vaccine-induced immunity by pyran can be shown to be specifically directed against the cell type used initially for vaccination.

MATERIALS AND METHODS

Animals. Adult male BALB/c × DBA/2 F1 (H-2^d); hereafter called B6D2F1) mice, 6 to 8 weeks of age and weighing approximately 25 g, were supplied by the Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Md. Mice were housed in plastic cages with air filter bonnets and fed Purina laboratory chow and water ad libitum.

Leukemias. The L1210 leukemia, syngeneic to DBA/2 (H-2^d) mice, has been carried in the transplantable ascites form in B6D2F1 mice for over 80 generations in this laboratory. The LSTRA murine leukemia was established by inoculation of BALB/c (H-2^d) mice with Moloney leukemia virus and has been carried in the transplantable ascites form in CD2F1 mice for over 180 generations in this laboratory. Both leukemias were diluted to the desired cell concentrations, using Eagle's minimal essential media containing 100 units penicillin per ml and 100 μg streptomycin per ml. Viability was determined by using trypsin blue exclusion test, and 10^4 tumor cells were inoculated i.p. in a volume of 0.2 ml/mouse as a challenge dose after vaccination.

Drugs. Pyran copolymer (NSC 46015) and several other pyran preparations of differing viscosities were kindly supplied by Dr. David Breslow of Hercules Research Center, Wilmington, Del. All pyrans were dissolved in sterile 0.9% NaCl solution and the pH was adjusted to 7.0 using 1.0 N NaOH. All compounds were administered i.p. to the mice in an injection volume equal to 1% of their body weight.

Vaccine Preparation. Tumor cells to be used for vaccine were adjusted to a concentration of 5.0 × 10^7 cells/ml in Eagle's minimal essential media with 100 units penicillin per ml and 100 μg streptomycin per ml. They were then inactivated by exposure to 5000 R X-radiation from a double-tube Westinghouse Quadrocondex X-ray machine and administered i.p. to the mice in an injection volume of 0.2 ml 7 days prior to live tumor challenge. Animals receiving both tumor cell vaccine and pyran on the same day received the vaccine 1 to 2 hr after pyran treatment in a separate injection. Systemic leukemia was never produced by inoculation of the vaccine preparation alone in control groups.

RESULTS

Potentiation of L1210 Vaccine by Pyran. The finding that concomitant administration of pyran copolymer and radiation-inactivated L1210 vaccine 7 days prior to live L1210 challenge produced striking numbers of animals resistant to the challenge and markedly enhanced the survival time of those animals that did succumb to the challenge is shown in Table 1. Animals that received L1210 vaccine alone, consisting of 1.0 × 10^7 X-irradiated L1210 ascites cells, all survived longer than 70 days, indicating that the tumor cells...
had been completely inactivated by 5000 R. All animals challenged with $1.0 \times 10^4$ L1210 ascites cells succumbed to the leukemia with an average survival time of 10.7 days. Pretreatment with L1210 vaccine or 25 mg pyran copolymer per kg 7 days before the L1210 challenge did not result in any significant increase in resistance to the leukemia. However, animals that received both pyran and L1210 vaccine 1 week before tumor challenge showed a remarkably potentiated immunity to the L1210 challenge with 43% totally resistant, and a large increase in average survival time among those that died of systemic leukemia. In addition, a smaller number of survivors of the 1st challenge were rechallenged 70 days later with $1.0 \times 10^4$ live L1210 cells, and almost all were immune. This strong synergistic effect seen with combined administration of pyran and L1210 vaccine was then evaluated in more detail in the following studies.

**Pyran and Vaccine Dosage Requirements.** To determine how sensitive the potentiation of L1210 vaccine by pyran was to pyran dosage, varying amounts of pyran were given, along with vaccine, to animals subsequently challenged with live L1210 cells. In Table 2, a significant increase in survival time was obtained with as little as $1.0 \text{ mg of pyran per kg}$, while $0.5 \text{ mg per kg}$ was little better than the controls. Optimal effects with significant numbers of animals immune to challenge were seen at doses between $2.5$ and $75 \text{ mg/kg}$. Higher doses were less effective and some drug toxicity was observed at $200 \text{ mg/kg}$. Clearly, the potentiation of the L1210 vaccine was dose dependent on pyran, but at the same time there was a wide effective dose range.

Table 2 also summarizes an experiment in which the dose of pyran was held constant at the optimal dose of $25 \text{ mg/kg}$, and the amount of L1210 vaccine given was varied from $10^4$ to $10^5$ cells/mouse. Again, the model was very sensitive to dosage with no vaccine potentiation apparent until a dose of $1.0 \times 10^6$ radiated cells was given, producing a significant increase in survival time. Pyran required $1.0 \times 10^7$ radiated L1210 cells to produce a large number of animals immune to L1210 challenge, with those that did die also

### Table 1

<table>
<thead>
<tr>
<th>L1210 vaccine (10^4 cells, i.p., Day —7)</th>
<th>Pyran copolymer (25 mg/kg, i.p., Day —7)</th>
<th>Survivors/dtotal</th>
<th>Survival time (days)</th>
<th>Rechallenge survivors/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>132/132</td>
<td>70</td>
<td>0/52</td>
</tr>
<tr>
<td>—</td>
<td>+</td>
<td>0/139</td>
<td>10.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>—</td>
<td>0/151</td>
<td>10.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>0/151</td>
<td>12.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>72/166</td>
<td>27.9 ± 1.0</td>
<td>31/34</td>
</tr>
</tbody>
</table>

* Animals scored for survival 70 days after L1210 challenge.
* Calculated from the individual days of death of those animals dying from systemic leukemia.
* Animals rechallenged with $10^6$ L1210 cells on Day 70.
* Average ± S.E.

### Table 2

<table>
<thead>
<tr>
<th>Pyran copolymer (i.p., Day —7)</th>
<th>Survivors/dtotal</th>
<th>Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0/10</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>0.5</td>
<td>0/10</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td>1.0</td>
<td>1/10</td>
<td>20.7 ± 2.7</td>
</tr>
<tr>
<td>2.5</td>
<td>7/10</td>
<td>NS</td>
</tr>
<tr>
<td>5.0</td>
<td>8/10</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>8/10</td>
<td>NS</td>
</tr>
<tr>
<td>25</td>
<td>9/10</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>6/10</td>
<td>NS</td>
</tr>
<tr>
<td>75</td>
<td>5/10</td>
<td>NS</td>
</tr>
<tr>
<td>100</td>
<td>3/10</td>
<td>30.0 ± 3.6</td>
</tr>
<tr>
<td>200</td>
<td>2/8</td>
<td>22.8 ± 5.1</td>
</tr>
</tbody>
</table>

* All animals received $1.0 \times 10^5$ radiated L1210 vaccine cells, i.p., on Day —7.
* All animals received pyran copolymer, 25 mg/kg, i.p., on Day —7.
* Animals scored for survival 70 days after challenge with $1.0 \times 10^6$ live L1210 cells, i.p., on Day 0 into B6D2F, mice.
* Calculated from the individual days of death of those animals dying from systemic leukemia.
* Average ± S.E.
* NS, not significant due to the large number of long-term survivors in the group.
* Two animals died of apparent drug toxicity without leukemia.
having a marked increase in survival time.

**Effect of Time of Pyran Administration.** The beneficial results in Tables 1 and 2 were obtained by giving pyran and the irradiated L1210 cells in separate injections 1 to 2 hr apart 1 week prior to live tumor challenge. This simultaneous method of injection was optimal for protection against L1210 challenge, as is evident in Table 3, in which pyran also was given several days before or after vaccination on Day –7. Pyran given 2 to 3 days before vaccination resulted in no protective effect. Slightly beneficial responses were obtained when pyran was given 1 day before or up to 4 days after vaccination, but clearly the most effective schedule was either simultaneous vaccine and pyran administration, or pyran injection 1 day after vaccination.

**Relationship of Vaccine Potentiation to Pyran Intrinsic Viscosity.** Previous reports have suggested a relationship between the intrinsic viscosity of pyran and its activity (2, 16) and toxicity (2). A similar correlation has also been noted for the poly(l) moiety of poly(l):poly(C) (15). Therefore, several different pyrans, prepared and purified to select a particular molecular weight more uniform than NSC 46015, were tested for comparative activity. In Table 4 they are listed according to increasing intrinsic viscosity, which is an indirect method of measuring molecular weight. The molecular weight average and intrinsic viscosity of NSC 46015, the compound used in all the other experiments reported here because of its clinical formulation and widespread usage, are shown for comparison. As is evident in Table 4, all the pyran preparations when used with vaccine were active, but no protection to challenge was afforded if they were used alone. Also, there is a trend toward more long-term survivors and longer average survival times as the intrinsic viscosity or molecular weight increases. Hence, the potentiating activity of pyran seemed to be related to the size of the polymer used.

**Specificity of Vaccine Potentiation by Pyran.** To determine whether the enhanced protection against tumor challenge seen with concomitant tumor cell vaccine and pyran

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### Table 3

**Effect of timing of pyran dosage on L1210 vaccine potentiation**

<table>
<thead>
<tr>
<th>Day of L1210 vaccine dosage (10⁶ cells, i.p.)</th>
<th>Day of pyran therapy (25 mg/kg, i.p.)</th>
<th>Survivors/total</th>
<th>Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day –7</td>
<td>Day –7</td>
<td>0/10</td>
<td>12.0 ± 0.7*</td>
</tr>
<tr>
<td>Day –7</td>
<td>Day –8</td>
<td>0/10</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>Day –9</td>
<td>Day –8</td>
<td>0/10</td>
<td>13.3 ± 0.4</td>
</tr>
<tr>
<td>Day –10</td>
<td>Day –3</td>
<td>2/29</td>
<td>17.8 ± 1.8</td>
</tr>
<tr>
<td>Day –6</td>
<td>Day –3</td>
<td>3/30</td>
<td>26.5 ± 2.3</td>
</tr>
<tr>
<td>Day –5</td>
<td>Day –3</td>
<td>8/20</td>
<td>23.8 ± 3.5</td>
</tr>
<tr>
<td>Day –7</td>
<td>Day –3</td>
<td>1/9</td>
<td>25.4 ± 3.5</td>
</tr>
<tr>
<td>Day –7</td>
<td>Day –3</td>
<td>1/10</td>
<td>17.6 ± 1.1</td>
</tr>
</tbody>
</table>

* Animals scored for survival 70 days after challenge with 10⁶ live L1210 cells, i.p., on Day 0 in B6D2F, mice.
* Calculated from the individual days of death of those animals dying of systemic leukemia.
* Average ± S.E.

### Table 4

**Effect of pyran viscosity on potentiation of L1210 vaccine**

<table>
<thead>
<tr>
<th>Pyran preparation (5 mg/kg, i.p., Day –7)</th>
<th>Intrinsic viscosity [η]</th>
<th>L1210 vaccine (10⁶ cells, i.p., Day –7)</th>
<th>Survivors/total</th>
<th>Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X18729-91</td>
<td>0.165</td>
<td>–</td>
<td>0/10</td>
<td>11.0 ± 0.3*</td>
</tr>
<tr>
<td>X18720-91</td>
<td>0.165</td>
<td>+</td>
<td>1/10</td>
<td>19.4 ± 0.9</td>
</tr>
<tr>
<td>X18571-92</td>
<td>0.30</td>
<td>–</td>
<td>0/10</td>
<td>10.3 ± 0.2</td>
</tr>
<tr>
<td>X18571-92</td>
<td>0.30</td>
<td>+</td>
<td>0/10</td>
<td>20.8 ± 2.8</td>
</tr>
<tr>
<td>X18720-71</td>
<td>0.70</td>
<td>–</td>
<td>0/10</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>X18720-71</td>
<td>0.70</td>
<td>+</td>
<td>3/10</td>
<td>28.6 ± 3.4</td>
</tr>
<tr>
<td>X18720-39B</td>
<td>1.08</td>
<td>–</td>
<td>0/10</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>X18720-39B</td>
<td>1.08</td>
<td>+</td>
<td>4/10</td>
<td>26.2 ± 2.3</td>
</tr>
<tr>
<td>X18802-32</td>
<td>1.58</td>
<td>–</td>
<td>0/10</td>
<td>11.7 ± 0.5</td>
</tr>
<tr>
<td>X18802-32</td>
<td>1.58</td>
<td>+</td>
<td>5/10</td>
<td>36.8 ± 5.4</td>
</tr>
</tbody>
</table>

* Polyelectrolyte run as the sodium salt in 0.05 M NaCl. NSC 46015 is equal to about M.W. 18,000 and has an intrinsic viscosity at about 0.76.
* Animals scored for survival 70 days after challenge with 10⁶ live L1210 cells, i.p., on Day 0 in B6D2F, mice.
* Calculated from the individual days of death of those animals dying of systemic leukemia.
* Average ± S.E.
administration was specific, a different mouse strain (CD2F1) was used that would accommodate 2 different leukemias. The L1210 and LSTRA leukemias produced similar average survival times in these mice using equal challenge doses (Table 5). Vaccination with either tumor alone was ineffective unless combined with pyran, which then produced significant numbers of animals resistant to challenge. Pyran also showed some protective effect against the LSTRA leukemia by itself. However, combined LSTRA vaccine with pyran treatment did not protect against live L1210 challenge, and L1210 vaccine with pyran did not protect against LSTRA challenge. Therefore, the markedly enhanced protective effect seen with pyran and vaccine was specific, in that animals were rendered immune to only the cell type originally used in the vaccine and were essentially as susceptible as controls to challenge from a different cell type.

DISCUSSION

We have reported the observation that concomitant administration of a polyanionic chemical, pyran, and a tumor cell vaccine, L1210, resulted in a strikingly enhanced immunity to subsequent L1210 challenge, whereas either agent alone was ineffective. This immune potentiation by pyran occurred despite the fact that L1210 is a poorly immunogenic tumor requiring multiple vaccinations to achieve even a slight degree of immunity (4). Furthermore, this was not a transient immune potentiation, since a high degree of resistance to challenge was present 70 days after the initial immunization (Table 1).

Although we have not as yet examined the mechanism of this potentiation by in vitro methods, certain information regarding the activity of pyran in this model system is provided by the reported in vivo results. In Table 2, a widely effective dosage range for pyran is seen, yet that for the vaccine was quite limited. In order for pyran to potentiate immunity, a certain critical antigenic mass was required. This was further shown in Table 3, where a gradual loss of activity of pyran occurred, the longer after vaccination that pyran was given. This finding was probably due to the diminishing antigenic mass, a result of natural host processing, available to pyran if it was given more than 24 hr after vaccination. Hence, the timing of pyran therapy in relation to the presentation of antigen was of great importance. A further aspect of the critical dependence on time was seen if pyran was given prior to vaccination. Little activity was present at 24 hr and none at 48 hr before vaccine injection, indicating that the pyran effect was almost entirely over 24 hr after its i.p. injection. Hence, in order for immunological stimulation to occur, pyran must be given at a time when the antigenic mass is maximal, and early or late dosage results in a great loss of activity. This observation partially explains why the time of pyran therapy was critical in a previous report, in which the inactivated antigenic mass was provided by chemotherapy of an established tumor (16).

The activity of pyran in this vaccine model seemed to be related to the molecular weight of the polymer, although all molecular weights were active to a certain degree. The meaning of this relationship is unclear at present but may have to do with the amount of negative charge on the copolymer. The similarity to findings already reported for poly(I):poly(C) is of interest and is being pursued further.

That this potentiation of the immunity induced by tumor cell vaccination was rather specific for challenge by the vaccinating cell type was shown in Table 5. In Line 12 of that table there appears to be some protection to challenge by LSTRA afforded by vaccination with L1210 and pyran treatment. However, when the average survival time is compared

<table>
<thead>
<tr>
<th>Pyran copolymer (25 mg/kg, Day -7)</th>
<th>Vaccine cell type (10⁶ cells, i.p., Day -7)</th>
<th>Challenge cell type (10⁶ cells, i.p., Day 0)</th>
<th>Survivors*/total</th>
<th>Survival timea (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>L1210</td>
<td>L1210</td>
<td>12/12</td>
<td>&gt;70</td>
</tr>
<tr>
<td>-</td>
<td>L1210</td>
<td>L1210</td>
<td>0/12</td>
<td>10.2 ± 0.2</td>
</tr>
<tr>
<td>+</td>
<td>L1210</td>
<td>L1210</td>
<td>0/12</td>
<td>11.0 ± 0.2</td>
</tr>
<tr>
<td>-</td>
<td>L1210</td>
<td>L1210</td>
<td>12/12</td>
<td>&gt;70</td>
</tr>
<tr>
<td>+</td>
<td>L1210</td>
<td>L1210</td>
<td>12/12</td>
<td>&gt;70</td>
</tr>
<tr>
<td>LSTRA control groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>LSTRA</td>
<td>LSTRA</td>
<td>12/12</td>
<td>70</td>
</tr>
<tr>
<td>-</td>
<td>LSTRA</td>
<td>LSTRA</td>
<td>0/12</td>
<td>11.2 ± 0.3</td>
</tr>
<tr>
<td>+</td>
<td>LSTRA</td>
<td>LSTRA</td>
<td>1/12</td>
<td>17.5 ± 0.3</td>
</tr>
<tr>
<td>+</td>
<td>LSTRA</td>
<td>LSTRA</td>
<td>7/12</td>
<td>15.4 ± 0.7</td>
</tr>
<tr>
<td>L1210-LSTRA cross challenge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>L1210</td>
<td>LSTRA</td>
<td>0/12</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>+</td>
<td>L1210</td>
<td>LSTRA</td>
<td>0/12</td>
<td>16.5 ± 0.3</td>
</tr>
<tr>
<td>-</td>
<td>LSTRA</td>
<td>L1210</td>
<td>0/12</td>
<td>10.1 ± 0.1</td>
</tr>
<tr>
<td>+</td>
<td>LSTRA</td>
<td>L1210</td>
<td>0/12</td>
<td>11.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Animals scored for survival 70 days after live tumor cell challenge in CD2F1 mice.
* Calculated from the individual days of death of those animals dying from systemic leukemia.
* Average ± S.E.
against Line 8 above, it is apparent that pyran alone could have accounted for the increased life-span. Therefore, although one cannot totally exclude the possibility that some cross-immunity occurred, there was none of significance compared to the control groups. More exhaustive attempts at cross-immunization would be necessary to demonstrate complete specificity, and possibly the apparent specificity seen here would break down if more similar tumor cell types were used. Further attempts at cross-immunization are being done at present, using 2 different leukemias, both of which carry the antigens of the Moloney leukemia virus. Although the study is not conclusive, pyran clearly augmented the immunity toward the vaccinating cell type as opposed to a different cell type and therefore behaved in a specific manner in this system. The sensitivity of this specificity has to be examined further.

In all of the reported studies the i.p. route of injection was followed for vaccine administration, pyran dosage, and tumor challenge. We have found that pyran seemed to be effective only by i.p. inoculation and that the administration of the vaccine and pyran mixed in a single injection actually increased the immunity even further (S. J. Mohr, unpublished results). Further experiments are necessary, however, to establish whether the immunity produced by pyran and vaccine therapy is effective against tumor challenge at distant sites or whether it is limited to the i.p. compartment. Also, this approach is being studied in application to established tumors.

B6D2F1 mice (H-2b) were primarily used for the model in these studies in the rejection of the L1210 tumor (H-2b). Because of this hemisyngeneic system, the possibility exists that pyran may have stimulated a phenomenon known as antiparent F1 hybrid histoincompatibility (24). Whether pyran enhanced the response of the F1 hybrid to parental cellular antigens or to tumor-associated antigens is unclear. Certainly, these 2 responses are not necessarily mutually exclusive. Preliminary experiments in our laboratory indicate that potentiation of the immunity toward L1210 by pyran also occurs in the DBA/2 mouse (H-2b), but to a lesser degree than in the B6D2F1 mice (S. J. Mohr, unpublished observations). Hence, it would seem that in this syngeneic system pyran stimulated a response to tumor-associated antigens, but this still does not exclude a stimulated antiparent response in the hemisyngeneic system. Both in vivo and in vitro studies are being performed to clarify this point further.

Results similar to those reported here have been seen for Bacillus Calmette-Guérin (8), Corynebacterium parvum (23), and endotoxin (20), and enhancement by pyran of the resistance to virus challenge following viral vaccination has also been reported (3). We have shown that pyran can be extended to neoplastic situations and therefore may be of benefit in those clinical situations in which biological immune modulation, currently in practice, is not feasible.

REFERENCES

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