ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1976, p. 28-33
Vol. 10, No. 1

Printed in U.S.A.

Antiviral Effect of Pyran Against Systemic Infection of Mice with Herpes Simplex Virus Type 2

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Received for publication 9 February 1976

The immunomodulator pyran markedly protected 5-week-old mice from lethal intravenous infection with herpes simplex virus type 2. The 50% lethal dose was increased almost 100-fold in pyran-treated mice as compared with controls. Although the protection was not as marked in older mice (10 and 16 weeks old), there was a significant increase in mean survival time. When the pathogenesis of herpesvirus disease was monitored in control and drug-treated mice, the effect of pyran was most evident in the spinal cord, where virus was recovered from 20 of 25 control mice and from only 6 of 25 pyran-treated mice. There was also a significant reduction in the titer of virus present, and virus appeared later in the spinal cord of pyran-treated mice than in control mice. The protective effect of pyran was observed only when the drug was administered 24 h before viral challenge, was seen after both intraperitoneal and intravenous injection, and was not due to direct inactivation of the virus.

Adequate antiviral chemotherapy is not available for herpesvirus infections such as herpes encephalitis, herpes zoster, or herpes labialis or genitalis. The results of recent clinical investigations with adenine arabinoside and phosphonoacetic acid have been equivocal (T. C. Merigan [ed.], J. Infect. Dis., in press). In certain clinical situations, treatment with related antiviral chemotherapeutic agents such as iododeoxyuridine (8), cytosine arabinoside (31), or neutral red (26) has produced adverse effects in terms of unacceptable toxicity or exacerbation of the viral disease itself. An alternate or adjuvant approach to the control of herpesvirus infections might be the use of immunomodulators. Therapy with immunomodulators as adjuvants with surgery or chemotherapy has proven more effective than either alone in inhibiting tumor growth (13). Treatment with the biological immunomodulator BCG has been reported to ameliorate recurrent herpes vaginalis in women (1). Prophylactic treatment of rabbits and mice with BCG resulted in some protection against systemic herpesvirus infection (3, 19). Protection could also be produced by the synthetic polyanions polyriboinosinic-cytidylic acid (9, 14) and chlorite-oxidized oxyamylose (4, 5). Another synthetic polyanion, pyran, a copolymer of maleic anhydride and divinyl ether, profoundly alters the reticuloendothelial system (25) and various immunological responses (2, 24) and induces interferon (21). Pyran has been shown to be an effective antiviral agent against several ribonucleic acid viruses, including vesicular stomatitis virus (12), foot-and-mouth disease virus (30), encephalomyocarditis (24) and mengovirus (20), ribonucleic acid leukemia viruses (10, 22, 29), and one deoxyribonucleic acid virus, vaccinia (11). We have previously demonstrated that pyran is protective against intravenous or intravaginal infection of mice with herpes simplex virus type 2 (HSV-2) (23). In the present study, we examined the effect of varying the route and the time of administration of the drug and monitored the effect of pyran on the pathogenesis of systemic herpesvirus disease.

(This paper was presented in part at the 75th Annual Meeting of the American Society for Microbiology, New York, 27 April to 2 May 1975.)

MATERIALS AND METHODS

Mice. Female BALB/c mice (Flow Research Animals, Inc., Dublin, Va., and Laboratory Supply, Indianapolis, Ind.) were received at 4 to 5 weeks of age and usually were used for experiments at 5 to 6 weeks of age.

Cells. HEp-2 cells (Microbiological Associates, Bethesda, Md.) and Vero cells (courtesy of Byron Murray) were grown in Eagle minimal essential medium with Hanks balanced salt solution (HMEM), 10% heat-inactivated fetal calf serum, and no antibiotics. For virus titrations, HMEM with 2% fetal calf serum, 1% methyl cellulose, 200 U of penicillin per ml, 100 μg of streptomycin per ml, and 1 μg of amphotericin B (Fungizone) per ml was used.

Virus. The virus used in this study was a recent human isolate identified as HSV-2 by the indirect hemagglutinin test (courtesy of John Stewart, Center for Disease Control, Atlanta Ga.). Virus was propagated in HEp-2 cells and had a titer of 5×10^6 plaque-forming units (PFU)/ml.

Virus titrations. Virus was titrated for PFU on cells grown in 24-well tissue culture plates (Linbro Chemical Co., New Haven, Conn.). The virus-infected cells were incubated for 2 days, and cells were stained with crystal violet to visualize plaques. Virus titrations were performed on HEp-2 cells, except where indicated.

Drugs. Pyran, a polyanionic copolymer of maleic anhydride and divinyl ether, lot XA 124-177, was obtained from David Breslow, Hercules, Inc., Wilmington, Del. The drug was solubilized in 0.15 N NaCl brought up to pH 7.0 with NaOH.

Animal experiments. Groups of 10 mice were injected with pyran intravenously (i.v.) (0.5 mg/mouse) or intraperitoneally (i.p.) (1.5 mg/mouse) and infected with virus 24 h later. These doses are similar to those reported effective against other viruses (20, 24) and are not toxic. The acute mean lethal dose (LD_{50}) is approximately 1.5 mg/mouse by i.v. injection. Mice were checked daily for mortality and the percent mortality was calculated. The majority of deaths occurred within 14 days after infection, and all had occurred by 25 days.

The mean survival time was calculated using 25 days as the day of death for the surviving mice. Statistical analysis of mortality data was performed using the chi-square test with Yates' correction factor, and mean survival time was evaluated by the Student's t test. For some experiments, groups of 10 animals were infected with several 10-fold dilutions of virus, and the PFU required for the LD_{50} was calculated by the method of Reed and Muench (28).

Pathogenesis studies. Mice were treated with 0.15 N NaCl or drug and infected as described. Groups of five control or drug-treated mice were sacrificed on various days after infection, organs were removed, and the individual samples were triturated in mortars. Blood, kidney, spleen, liver, spinal cord, cerebellum and brain stem, and cerebrum were collected from each animal, and the clinical status of the animal (well, hunched with ruffled fur, paralyzed, moribund) was noted. Ten percent (wt/vol) suspensions of all organs, except spinal cord and blood, were prepared in viral diluent containing Hanks balanced salt solution, 0.5% gelatin, 0.25% lactalbumin hydrolysate, and antibiotics. Spinal cord was prepared as a 5% suspension, and clotted blood was prepared as a 33% suspension. Specimens were centrifuged at $1,000 \times g$, and the supernatant fluids were frozen at −70 C until the separate specimens from individual mice were assayed for virus by plaque formation on Vero cells.

RESULTS

Antiviral action of pyran in mice of various ages. A marked age-related resistance to systemic infection with HSV has been noted to occur in mice between birth and 4 weeks of age (15, 16). Experiments were performed to determine whether this age-related resistance continued to increase in early adulthood and to follow the antiviral effects of pyran during this period. The LD_{50} of HSV-2 increased 50-fold between 5 and 10 weeks of age and remained relatively constant thereafter (Table 1). Prophylactic treatment with pyran markedly protected 5-week-old mice from HSV-2 lethality; the LD_{50} was increased 80-fold, from $10^{1.4}$ PFU in control animals to $10^{3.3}$ PFU in pyran-treated animals. Although the protective effects of pyran were not as marked in the 10- and 16-week-old mice, the LD_{50} was increased four- to eightfold, and the mean survival time of mice was significantly increased over controls. Although the older mice were larger, the milligrams per kilogram dose ranged from only 25 to 33 mg/kg, both dosages which we have shown to be effective in older mice.

Effect of pyran on HSV-2 pathogenesis. To follow quantitatively the course of HSV-2 infection in control and drug-treated 5-week-old animals, organs from individual mice whose clinical status was noted were collected at various times during infection and assayed individually for virus. Groups of 10 control and drug-treated mice were followed for mortality. In this experiment prophylactic i.v. treatment with pyran reduced the mortality from $^8/_{10}$ in control mice to $^1/_{10}$ in pyran-treated mice.

No virus was detected in any samples of blood, spleen, or liver assayed between days 1 and 13 after i.v. virus infection. However, low levels of virus (less than 10^3 PFU/g) were sporadically detected in the kidney in 2 of 25 samples from control mice and in 6 of 25 samples from pyran-treated mice harvested on days 6 to 13 after infection. Amounts of virus in the cerebellum-brain stem reached peak mean titers of less than 10^2 PFU/g between days 8 and 11 in both control and drug-treated groups. A titer of 10 PFU/g of cerebral tissue was observed in only one drug-treated mouse on day 11 after infection.

The effect of pyran treatment on the pathogenesis of HSV-2 was most evident in the spinal cord. The geometric mean titers of virus present in the spinal cord were significantly reduced in drug-treated as compared with control mice on days 6 through 10 after infection (Fig. 1). Furthermore, virus was recovered from the spinal cord in 20 of 25 control mice and from only 6 of 25 pyran-treated mice that were sacrificed between days 6 and 13 after virus infection. Virus also appeared later in the drug-treated mice. A level of 3×10^3 PFU/g of tissue was observed on day 6 in control animals,

TABLE 1. *Antiviral action of pyran in mice of various ages*

Treatment	5-week-old mice		10-week-old mice		16-week-old mice	
	LD_{50}[a]	MST ± SE[b]	LD_{50}	MST ± SE	LD_{50}	MST ± SE
Control	1.4	9.8 ± 1.0	3.1	13.4 ± 1.7	3.5	20.5 ± 1.5
Pyran[c]	3.3	20.6 ± 2.1[d]	4.0	24.5 ± 2.3[d]	4.1	>25.0[d]

[a] LD_{50} expressed as $\log_{10}$ PFU per 0.2 ml of inoculum per mouse.
[b] Mean survival time ± standard error in mice inoculated with $10^{3.3}$ or $10^{3.6}$ PFU/mouse.
[c] Pyran (0.5 mg/mouse, i.v.) was inoculated 24 h before i.v. HSV-2 challenge.
[d] $P < 0.05$.

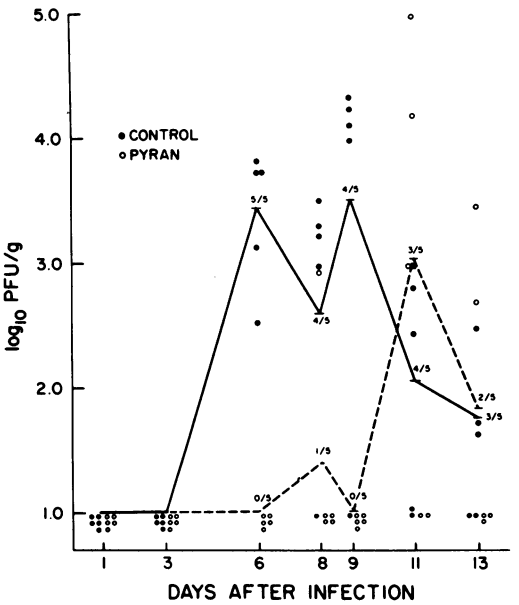

FIG. 1. *Effect of pyran on growth of HSV-2 in spinal cord. Mice were treated with 0.15 N NaCl or pyran and infected i.v. with HSV-2 24 h later; five mice in each group were sacrificed on the days indicated, and virus present in the spinal cord was determined by plaque formation on Vero cells. Symbols: ●, control mice; ○, pyran-treated mice. The bars represent the geometric mean virus titer of all five mice, with the number of positive mice indicated.*

whereas a similar level was not obtained until day 11 in drug-treated mice. The inhibitory effect of pyran was even greater than the data indicate. Because we wanted to evaluate the ability to correlate clinical illness with HSV-2 present in the central nervous system, a particular effort was made to include both sick and apparently well mice in each group when organs were harvested. In general, the presence of HSV-2 in the spinal cord correlated well with the clinical status of the animal. Except on day 6, when all control animals appeared well but exhibited virus in the central nervous system, virus was only recovered from mice with clinical illness.

Lack of direct inactivation of HSV-2 by pyran. Inhibition of HSV-2 could occur by direct inactivation of the virus. Pyran has been reported to inactivate certain viruses (20). However, when HSV-2 was mixed with varying concentrations of pyran in vitro for 1 h at 36 C, there was little loss in infectivity (Table 2). Concentrations of 12.5 mg of pyran per ml were required to reduce the virus titer one $\log_{10}$. Since mice were treated with 0.5 mg, it seemed unlikely that direct inactivation of the virus could account for the antiviral effect of the drug. However, it was possible that pyran could be altered in vivo to an active inhibitory form. Serum and 10% spleen homogenates were prepared from mice 24 h after i.v. injection with pyran, at the time when mice were protected in vivo against i.v. challenge with virus. These serum and spleen homogenates did not directly inactivate HSV-2 in vitro (data not shown).

Effect of varying the route of administration of pyran. The antiviral effect of polyanions has been reported to be a primarily local effect (20). Mice were treated with pyran either i.v. (0.5 mg) or i.p. (1.5 mg) and infected 24 h later

TABLE 2. *Effect of incubation of pyran and HSV-2 in vitro*[a]

Final concn of pyran (mg/ml)	HSV-2 titer (PFU/ml)
Expt 1	
None	4.8×10^6
1.25	2.5×10^6
2.5	4.8×10^6
Expt 2	
None	5.5×10^5
1.55	3.1×10^5
3.12	1.9×10^5
6.25	3.2×10^5
12.5	4.6×10^4
25.0	1.1×10^3

[a] Varying concentrations of pyran were mixed with a constant amount of HSV-2, the mixture was incubated for 1 h at 36 C, and the titer of residual virus was determined. Virus was incubated alone in diluent for the control.

with HSV-2 i.p. Administration of the drug by either route was effective in reducing mortality from HSV-2 infection (Table 3) and in increasing the mean survival time of mice (data not shown).

Effect of varying the time of administration of pyran. The protective effect of pyran was limited with respect to the time of drug administration relative to viral inoculation. Marked protection against virus lethality was observed only when pyran was administered 24 h before viral challenge (Table 4).

Effect of pinworm infestation on HSV-2 infection and pyran protection. Evidences of either pinworm infestation or *Pasteurella pneumotropica* infection of the brain were observed in certain shipments of mice. To determine whether pinworm infestation affected experiments, pinworm-infested and pinworm-free mice were treated with pyran and infected with HSV-2. Half of one shipment of mice was treated orally with piperazine (11 mg of piperazine citrate per ml of drinking water) for 2 weeks until they were found to be completely pinworm free, whereas the pinworm-infested mice were housed normally but kept isolated from other mice. Pinworm infestation did not markedly affect the HSV-2 infection (Table 5). Although pyran appeared to be equally effective in pinworm-free and -infested mice at day 17 after virus infection, subsequent late deaths occurred in the pyran-treated, pinworm-infested mice. The effects of *Pasteurella* infection could not be directly ascertained since the organism could not readily be passed serially in mice. However, this experience emphasizes the role intercurrent infections may play in modifying drug-virus-host interactions.

TABLE 3. *Effect of route of pyran administration on protection against HSV-2 infection*[a]

Route of pyran	No. of dead mice/total in expt:			Total	*P* value
	1	2	3		
None	8/10	7/10	10/10	25/30	
i.p.	2/10	1/10	NT	3/20	<0.001
i.v.	NT	3/10	5/10	8/20	<0.005

[a] Pyran (1.5 mg i.p. or 0.5 mg i.v.) was inoculated 24 h before i.p. challenge of 5-week-old mice with $10^{2.3}$ PFU of HSV-2. NT, Not tested.

TABLE 4. *Effect of varying the time of administration of pyran on protection against HSV-2*

Time of pyran treatment (h)[a]	No. of dead/total	MST ± SE[b]
None	20/20	9.0 ± 0.6
−168	10/10	8.4 ± 0.6
−96	9/10	12.2 ± 1.1 (<0.025)[c]
−48	10/10	9.2 ± 0.9
−36	10/10	11.3 ± 1.0 (<0.05)
−24	5/10 (<0.005)	16.0 ± 1.3 (<0.005)
−12	7/7	10.9 ± 0.6 (<0.05)
−2	10/10	9.3 ± 0.6
+24	8/10	12.2 ± 1.4 (<0.05)

[a] Pyran (0.5 mg/mouse) was inoculated i.v. at varying times between −168 to +24 h with respect to the time of i.v. challenge of 5-week-old mice with $10^{2.8}$ PFU of HSV-2.

[b] Mean survival time ± standard error.

[c] Numbers in parentheses represent *P* values.

TABLE 5. *Effect of pinworm infestation on HSV-2 infection and pyran protection*[a]

Pinworm infestation	Pyran treatment	No. of dead/total on:		MST ± SE[b]
		Day 17	Day 20	
−	−	14/15	14/15	9.6 ± 0.8
−	+	8/15[c]	8/15[c]	15.0 ± 1.2[c]
+	−	14/15	14/15	9.9 ± 0.8
+	+	7/14[c]	10/14	15.9 ± 1.0[c]

[a] Pinworm-free and -infested 6-week-old mice were treated i.v. with pyran (0.5 mg/mouse) 24 h before i.v. challenge with HSV-2 ($10^{3.0}$ PFU).

[b] Mean survival time ± standard error.

[c] $P < 0.05$.

DISCUSSION

Several studies have investigated the antiviral activity of the immunomodulator pyran against ribonucleic acid viruses (12, 20, 30). The mode of antiviral action of pyran has not been completely elucidated. Prophylactic administration was essential for activity against HSV-2 infection, as has been previously reported for activity against mengovirus infection (20). Merigan and Finkelstein (20) showed that 1 to 5 mg/ml reduced plaque formation by mengovirus, vesicular stomatitis virus, and vaccinia virus; the authors concluded that direct virus inactivation was unlikely to account for the in vivo protection. With the HSV-2 used in the present studies, a dose of 10 mg/ml was required to reduce virus titer by one $\log_{10}$. These results are consistent with the previous data. The slight difference in amount of drug required for direct inactivation may reflect differences in viruses or the source of drug. Pyran has been demonstrated to be a potent inhibitor of ribonucleic acid-dependent deoxyribonucleic acid polymerase in vitro and to slightly inhibit alpha- and beta-mammalian polymerases (27). The necessity for prophylactic drug treatment is inconsistent with a mode of action involving

either direct virus inactivation or inhibition of virus replication. Furthermore, the systemic as well as local action of the drug argues against local drug effects.

In view of the known immunomodulating activity of the compound, enhancement of host resistance responses against the virus appears likely. The pathogenesis data indicated that pyran inhibited spread of the virus into the central nervous system. Similar to reports by Kern et al. (18), we have been unable to define completely the early pathogenesis of HSV-2 in the visceral organs in adult mice. However, pyran appears to reduce this early virus production, since mice treated with pyran who survive infection with HSV are often less resistant to rechallenge than are untreated mice (M. C. Breinig, P. F. Cline, and P. S. Morahan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, E88, p. 78).

It would be tempting to suggest interferon involvement in this early antiviral effect. However, there is no compelling evidence for a significant role for interferon in the antiviral effects of pyran. We have previously demonstrated that the antiviral effect of pyran against encephalomyocarditis virus was not correlated with induction of interferon (24). Similar conclusions were reached by Billiau et al. concerning the antiviral action of another polyanion, chlorite-oxidized oxyamylose, against mengovirus infection (5). The authors postulated that the antiviral effect might be related to macrophage activation in the peritoneal cavity. The fact that herpesviruses are generally among the most resistant to the antiviral effects of interferon also argues against an important role for interferon.

Pyran is an immunoadjuvant for certain humoral immune responses (2). However, we have seen no evidence for increased neutralizing antibody production in pyran-treated as compared with untreated mice (Breinig et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, E88, p. 78). Cell-mediated immunity has been implicated as important in recovery of animals from primary herpesvirus infections (7, 32), but we have shown that pyran was effective against HSV-2 in mice depleted of thymus-derived lymphocytes by adult thymectomy, lethal irradiation, and bone marrow reconstitution (23). These data suggest that pyran may exert its antiviral activity not through modulation of specific immunological responses toward HSV-2 but by activating macrophages for enhanced nonspecific antiviral activity. The increased efficacy of the drug in 5-week as compared with 15-week-old mice may have some bearing on this point. Hirsch et al. (15) have demonstrated that macrophages from young mice are immature in regard to resistance to HSV replication in vitro. Blaese (6) has demonstrated that a variety of immunomodulating agents such as BCG and polyriboadenylic-uridylic acid can cause immature macrophages to develop competence for immunological helper function. Pyran treatment, therefore, may enable immature macrophages to express antiviral resistance. Other evidence for a role for activated macrophages comes from observations that pyran-activated peritoneal cells exhibit cytotoxicity for tumor cells (17) and inhibit HSV-2 and vaccinia virus growth in vitro (unpublished data). However, a definitive role for macrophages activated by pyran in the inhibition of HSV-2 replication in vivo remains to be established.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant CA 16193 from the National Cancer Institute, grant IN-105A from the American Cancer Society, and Public Health Service training grant AI 00382 from the National Institute of Allergy and Infectious Diseases. PSM is a recipient of Public Health Service research career development award AI 70863 from the National Institute of Allergy and Infectious Diseases.

The technical assistance of Paul F. Cline is greatly appreciated.

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