

Mouse Resistance Against Foot-and-Mouth Disease Virus Induced by Injections of Pyran

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Received for publication 12 August 1970

Mouse resistance to foot-and-mouth disease virus (FMDV) was induced by intraperitoneal injections of pyran copolymer. A biphasic pattern of protection occurred with greatest resistance 4 and 48 hr after injection of this polyanion. Viremia was not detectable in pretreated mice challenge-exposed with FMDV. Incubation of virus with pyran did not alter viral infectivity in mice or tissue culture. Serum interferon was demonstrated 1 and 2 days after pyran administration.

Limited protection against foot-and-mouth disease virus (FMDV) after injection of RNA extracted from yeast was observed in 1960 (8, 33). More recently, a synthetic double-stranded polynucleotide (poly I:C) induced resistance, presumably related to interferon induction, against infection by FMDV in mice (19, 30). As reported for other viruses (18, 22), maximal protection against FMDV after poly I:C injection occurred for only a limited time. Induction of a prolonged antiviral state would be a necessary requisite in the consideration of an agent for possible use in the control of foot-and-mouth disease.

A number of other nonviral synthetic compounds of defined composition have antiviral activity which, in part, is probably related to interferon-mediated protection (9, 11, 12, 20-23, 26). Several of these agents induce long-lasting effects (9); notably, divinyl ether-maleic anhydride copolymer (pyran)-induced resistance may persist for 2 months (10, 22). Recent reports suggested that protection of adult mice (19) and guinea pigs (R. Sellers, *personal communication*) against FMDV could be attained by pretreatment with pyran copolymer.

This communication reports that resistance against FMDV infection in infant mice after pyran injection is biphasic and 1 week in duration.

MATERIALS AND METHODS

Virus. Foot-and-mouth disease virus, type Asia-1 (FMDV, Asia-1) was passaged five times in primary

bovine kidney cell (BKC) cultures. The stock virus, frozen at -70°C , had a titer of $10^{6.99}$ plaque-forming units (PFU)/ml on BKC and $10^{7.05}$ LD₅₀/ml in infant mice. Dilutions of virus were prepared in Hanks balanced salt solution supplemented with 0.5% lactalbumin hydrolysate and 2% bovine serum. The preparation and characteristics of the other FMDV types have been described elsewhere (29).

Pyran. Stock solutions of pyran (XA 124-177, molecular weight 27,000; Hercules, Inc., Wilmington, Del.) were prepared in phosphate-buffered saline (PBS) with 0.1 M magnesium and calcium ions (19). To hasten the dissolution of pyran, gentle heating (45°C) was occasionally employed. The pH was adjusted to 7.2 by using 6 N NaOH after which the pyran solution was sterilized by filtration through 0.45- μm Nalgene disposable filters.

Mice. All mice were produced at this laboratory. They were descendants of a Rockefeller H strain originally brought to this laboratory approximately 14 years ago. Details of the random mating procedure are given elsewhere (6). Infant mice of the Rockefeller H strain are much more susceptible to FMDV than adult mice (7), and consequently 5- to 9-day-old mice were used in most experiments.

Mouse resistance. Dilutions of pyran were prepared in PBS and intraperitoneal (ip) mouse injections were made (0.03 ml for infants, 0.1 ml for older mice). At various intervals thereafter, mice were given ip injections of virus. In some experiments a constant virus challenge dose was used (100 LD₅₀ per mouse) and resistance was measured as per cent of survival (30). Other tests were performed by parallel titrations of virus in treated or untreated mice. In these cases, a protective index was calculated as the log₁₀ difference in median lethal dose (1). Mouse survival was determined on the 7th day after virus injection. A survival rate of at least 50% or a protective index of at least 1 log was considered indicative of induced resistance.

Interferon assay. Serum interferon levels after injection of 120 mg of pyran per kg were determined as described elsewhere (30). The interferon titer was ex-

¹ Part of this work was done while in pursuance of a Post-doctoral Resident Research Associateship of the Agricultural Research Service and the National Academy of Sciences, National Research Council.

pressed as the reciprocal of the highest serum dilution which yielded 50% protection against the cytopathic effect of 100 LD₅₀/ml of FMDV, Asia-1 in primary mouse kidney cultures.

RESULTS

Induction of mouse resistance with pyran. The data presented in Table 1 indicate that resistance in infant mice against lethal doses of FMDV could be induced by pyran injection and suggest that the extent of protection increased as the interval between injection of pyran and virus increased. When injections of FMDV were made at various intervals after treatment with pyran, two peaks of maximal resistance were observed (Fig. 1). The peak which occurred 4 hr after pyran administration was short-lived, whereas the second was longer and followed a 48- to 72-hr induction period. A slightly lower protective effect continued until at least the 7th day (168 hr) and then declined.

Serum interferon. Little or no interferon was detected in mouse serum until 18 hr after ip injection of pyran (120 mg/kg). Titers ranging between 32 and 128 were determined in serums obtained after 1 to 2 days of induction but not in serum samples taken later.

Mouse age and virus type. Induction of resistance was not restricted to infant, 5- to 9-day-old mice (Table 2). Mice, 8, 21, and 34 days old, were given injections of pyran, and virus titrations were performed 2 and 7 days later in treated and untreated groups. The decreasing susceptibility to FMDV with increasing mouse age is evident in the control group and the data presented in Fig. 1. Protective indices for each age group with both induction periods demonstrate comparable pyran-induced resistance.

The results given in Table 3 clearly demonstrate that pyran stimulated resistance in infant mice against the seven FMDV types tested.

TABLE 1. Induction of resistance in mice as a function of time between intraperitoneal injections of pyran and 100 LD₅₀ FMDV, Asia-1

Concn of pyran (mg/kg)	No. of mice alive 7 days after virus injection			
	1 ^a	2	3	4
120	3 ^b	10	10	6
60	4	7	9	5
30	1	8	5	3
15	0	2	3	7
7.5	0	3	2	2
0	0	0	0	0

^a Days between injection of pyran and virus.

^b Total of 10 mice per group.

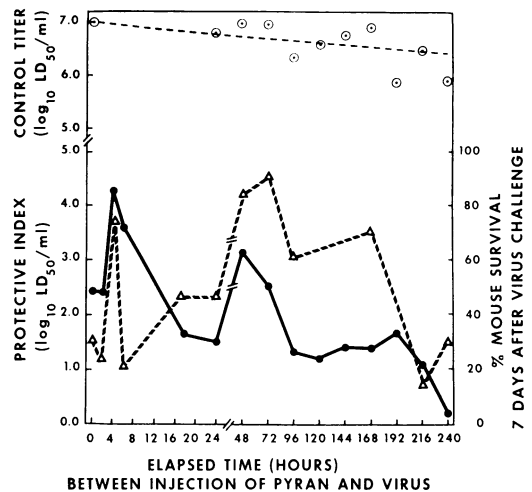


FIG. 1. Resistance of infant mice to FMDV observed at various intervals after injection of 120 mg/kg of pyran. Per cent survival (broken line) calculated 7 days after injection of 100 LD₅₀ FMDV, Asia-1. Protective index (solid line) calculated as the log₁₀ difference between virus titrations obtained from pyran-injected or control mice. The results of daily titrations of virus in control mice are given at the top of this figure.

TABLE 2. Virus titer end points observed after pretreatment with 120 mg of pyran per kg in mice of different ages

Age ^a (days)	Induction time ^b	Virus titer (log ₁₀ LD ₅₀ /ml)		
		Control	Treated	PI ^c
8	2	7.0	4.3	2.7
	7	6.9	5.6	1.3
21	2	6.2	2.4	3.8
	7	5.5	1.6	3.9
34	2	3.9	1.9	2.0
	7	2.9	1.6	1.3

^a Age when injected with pyran.

^b Days between injection of pyran and virus.

^c Protective index calculated as log₁₀ difference in titer between control and treated.

The survival and protective index experiments were done with different randomized groups of mice and are not strictly comparable.

Virus inactivation. Stock virus and pyran, in concentrations of 0.05 to 5 mg/ml, were mixed and incubated (22), and viral infectivity was subsequently assayed in mice and BKC cultures. The results of one such experiment with 2 mg of pyran per ml are given in Table 4. At this level of pyran or at the others tested no direct inhibition of FMDV was observed.

Comparative viremias. Viremia assays were performed to correlate the survival patterns with other biological parameters. The results of a typical experiment (Table 5) indicate that no virus was detected in plasma samples from mice

preinjected with pyran, whereas more than 5 logs of virus per ml were recovered from non-treated control animals.

DISCUSSION

The antitumor, antiviral, and antibacterial activities of polyanions, including pyran, have been reviewed recently (26, 36; W. Regelson and A. Munson, *Ann. N.Y. Acad. Sci.*, *in press*). Pyran induces interferon (9, 10, 19, 20, 22, 23, 25, 27), stimulates the immunological response (5; W. Regelson, *Int. Symp. Interferon, in press*), and produces a biphasic phagocytic reticuloendothelial response with inhibition followed by stimulation of liver and spleen cells (W. Regelson et al., *Int. Symp. Interferon, in press*). Evidence of toxic effects after pyran administration have been reported (22, 23, 25). Because of these varied effects, mechanisms other than interferon induction must be considered in interpreting pyran-induced resistance (9, 10, 22, 25, 28).

Pyran directly inhibits mengovirus, vesicular stomatitis virus, Friend leukemia virus, Semliki Forest virus, and echovirus 9 (10, 12, 22, 25) and to a lesser extent vaccinia virus (20), and yet there was no inhibition of plaquing efficiency or reduction in lethality for infant mice after incubating FMDV with this polymer (Table 4). Sellers (*personal communication*) was similarly unable to demonstrate interaction of FMDV with pyran. The relatively high protective index against FMDV infection observed immediately after pyran injection (Fig. 1) probably does not therefore reflect direct inhibition, although such interactions may have occurred within the peritoneal cavity of the mouse.

Statolon, synthetic polynucleotides, and lipopolysaccharides may initiate the release of "preformed" interferon (39-41) or, as recently postulated by Ho and Ke (16), the induction of "pre-interferon." These investigators suggest that an antiviral state is maintained after stimulation with agents similar to polyribonucleotides by the retention of preinterferon within the cell. Pyran-like endotoxin (3, 13, 24) may damage cell membranes. Polynucleotides associated with such cellular destruction (14, 25) might be the initiators of a preinterferon state reflected by the resistance found with simultaneous injections of pyran and FMDV (Fig. 1).

The transient antiviral state occurring 4 hr after pyran administration (Fig. 1) may also be due to localized preformed interferon or interferon-forming subunits, or it may represent the conversion of preinterferon to interferon (16). The subsequent synthesis of new

TABLE 3. Comparative resistance of infant mice treated with pyran and injected with different FMDV types

Virus type	Per cent survival ^a	PI ^b
Sat 1	80 ^c	4.4
Sat 2	85	5.4
Sat 3	95	3.6
Asia-1	95	3.2
A	83	3.6
C	50	2.7
O	75	3.2

^a Challenge dose was 100 LD₅₀, 48 hr after 120 mg/kg of pyran.

^b Protective index; virus titrations 48 hr after 120 mg/kg of pyran.

^c Total of 20 treated mice per group; no controls alive 7 days after virus injections.

TABLE 4. Comparative FMDV titers after incubation of virus with 2 mg of pyran per ml

Reaction mixture	BKC ^a titer (log ₁₀ PFU/ml)		Mouse titer at 1.5 hr (log ₁₀ LD ₅₀ /ml)
	1.5 hr ^b	20 hr	
Virus ^c control, 4 C.....	6.81	5.94	6.40
Virus and pyran, 4 C.....	7.13	5.89	6.50
Virus control, 37 C.....	6.68		6.02
Virus and pyran, 37 C.....	6.73		5.98

^a BKC, bovine kidney cell; PFU, plaque-forming units.

^b Hours of incubation before titration.

^c FMDV, Asia-1.

TABLE 5. Plasma virus titers in 14-day-old mice injected intraperitoneally with 120 mg of pyran copolymer per kg 48 hr before virus injection

Time after virus injection ^a (hr)	Virus titer in plasma ^b	
	Control	Pyran
6	0	0
24	5.72	0
48 ^c	5.61	0

^a Challenge dose: 10⁸ infant mouse LD₅₀ FMDV, Asia-1.

^b Log₁₀ plaque-forming units per milliliter.

^c Only 20% untreated control mice alive 48 hr after virus challenge.

interferon may be reflected in the demonstration of this viral inhibitor in serum samples obtained 24 to 48 hr after injection of pyran, which corresponds to the second (48 hr) peak of resistance (Fig. 1). That low doses of pyran were able to stimulate resistance only after an induction period of 2 to 3 days (Table 1) supports the idea that the synthesis and release of new interferon may be necessary for this longer protective period. Interferon was not detected in serum samples taken 3 or more days after pyran administration, an observation which agrees with those of Merigan (20) and Merigan and Finklestein (22).

The inhibition of FMDV multiplication in adult mice interdermally injected with complete Freund's adjuvant 10 and 3 days before ip injections of virus has been reported (15). Whereas viremia was absent in 65% of the mice pretreated with Freund's adjuvant, pyran pretreatment totally inhibited viremia (Table 5). Similar inhibition was reported when statolon was injected 24 hr before aerosol challenge by Columbia SK virus (1) or ip Mengovirus injection (31, 32).

Although pyran and other polyanions may block virus adsorption or release (12, 34), stimulation of the reticuloendothelial system may be the prime mechanism whereby the resistant state is manifested (15, 22, 24-28). A number of interferon-inducing agents appear to have adjuvanting effects on antibody titers (2-4, 14, 17, 35, 37, 38). Freund's adjuvant, which did not induce interferon but which stimulated subsequent virus-induced interferon, delayed the appearance of neutralizing antibody (15), an observation further implicating the involvement of the reticuloendothelial system in both interferon and immune responses. The exact mechanism whereby resistance is conferred must await the results of additional experimentation, however.

ACKNOWLEDGMENTS

The author thanks E. H. Thiel for his technical assistance and R. G. Mraz of Hercules, Inc., for supplying the pyran copolymer.

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