Sensitization to Endotoxin by Pyran Copolymer (35619)

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The synthetic polyanion, pyran copolymer, (a divinyl ether maleic anhydride copolymer, pyran) produces a marked biphasic response in the functional activity of the reticuloendothelial system (RES) (1). Pyran possesses antitumor (2) and antiviral activity, induces interferon (3), enhances immunologic responsiveness (4), protects mice from certain microorganisms, and depresses the microsomal enzymes which metabolize hexobarbital (5).

It is the purpose of this report to show that pyran sensitizes mice to the endotoxin of *Salmonella typhosa* in similar fashion to other agents which alter reticuloendothelial function. This sensitivity to endotoxin produced by pyran remains throughout both the depressed and stimulated phases of phagocytic RES activity. Furthermore, the sensitivity does not seem to be related to the action of pyran on the microsomal enzymes which are essential for the metabolism of hexobarbital and antipyrine. Stimulation of these enzymes in pyran-treated mice with chlorcyclizine reverses the enzyme inhibition, yet does not alter the sensitivity to endotoxin. Since pyran is now in clinical trial as an anticancer agent, its usefulness along with other potentially useful nonspecific host stimulators may be retarded until this endotoxin sensitivity is averted.

Materials and Methods. New York State Laboratory and Research, Albany (NYLAR-A) male mice, weighing between 20 and 25 g, were used throughout. All mice were maintained on Purina Laboratory Chow and tap water *ad libitum* prior to and during the experimental period.

Pyran copolymer, NSC 46015 (Hercules Company) with an average molecular weight of 18,000 was administered intravenously daily for 2 days in a dose of 25 mg/kg. Chlorcyclizine hydrochloride (Abbott Laboratories) was injected intraperitoneally in a dose of 25 mg/kg daily for 3 days. Heparin, sodium (Organon Inc.) was given subcutaneously in a dose of 2000 units/kg. *Lipopolysaccharide W., Salmonella typhosa* 0901 (Difco Laboratories) was suspended in 0.15 *M* sodium chloride and administered intravenously. The acute toxicity of endotoxin was determined by the method of Litchfield and Wilcoxan (6). Mice were observed for 72 hr after endotoxin for mortality.

RES activity was measured by the vascular clearance rate of ^{51}Cr sheep erythrocytes (sRBC). Liver, spleen and lung uptake of sRBC was determined 60 min after sRBC injection (7). To test for the RES stimulation, mice were injected intravenously with 10^6 *Diplococcus pneumoniae* or 10^8 *Staphylococcus aureus* in 0.2 ml of brain–heart infusion broth at the time when the vascular clearance rate was enhanced.

The duration of anesthesia induced with hexobarbital was used to determine the effect of a drug on the *in vivo* metabolism of hexobarbital. Eighty mg/kg of the racemic mixture of sodium hexobarbital (Evipal Special Chemicals Department, Winthrop Laboratories) was administered intravenously via the lateral tail vein. The time between injection of the barbiturate and the time when the animals could right themselves 3 times in 30 sec was the measure of the hexobarbital sleeping time.

In vitro metabolism of hexobarbital and antipyrine by the 9000*g* liver supernatant fraction was performed essentially after the method of Rubin *et al.* (8). One μmole of hexobarbital and 10 μmoles of antipyrine were added to the incubation flasks.

TABLE I. Vascular Clearance and Organ Uptake of ^{51}Cr sRBC in Pyran-Treated Mice.[a]

Group	Endotoxin LD_{50}(mg/kg)	$T/2$ (min)	% Injected dose/total organ		
			Liver	Spleen	Lung
			1 Day postpyran copolymer		
Saline	45	13.1 ± 0.8	34.8 ± 2.1	10.4 ± 0.6	2.0 ± 0.1
Pyran	0.3[c]	30.7 ± 2.6[c]	5.2 ± 1.0[c]	7.4 ± 1.1[c]	4.4 ± 0.1[c]
			7 Days postpyran copolymer		
Saline	45	13.1 ± 1.0	37.5 ± 3.0	11.7 ± 1.0	1.6 ± 0.4
Pyran	0.6[c]	5.2 ± 0.5[c]	56.6 ± 1.9[c]	14.2 ± 1.6[c]	5.7 ± 0.6[c]

[a] Values expressed as mean ± SE except for LD_{50}. Pyran administered iv in a dose of 25 mg/kg for 2 days.

[b] Ten mice used for RES function and 50 mice used for determination of LD_{50}.

[c] $p < .05$.

Results. Twenty-four hr after the last of two daily injections of pyran, there was a 134% prolongation in the vascular clearance rate and an 85% decrease in hepatic uptake of sRBC as compared to control mice (Table I). Splenic uptake was depressed 29% while the pulmonary localization was increased 120%. During this RES depressed state the mice were 150 times more sensitive to the lethal effects of endotoxin. Seven days after the last pyran injection, the mice showed a marked stimulation of RES activity as manifested by increases in uptake of sRBC of 60% in the liver, 21% in the spleen, and 256% in the lung. These mice not only showed increased phagocytic activity but increased resistance to 10^6 *D. pneumoniae* and 10^8 *S. aureus* (Table II). At this time period the mice were 74 times more sensitive to endotoxin (Table I).

A dose of 25 mg/kg of pyran was required to produce the sensitization to endotoxin. A dose of 12.5 and 6.2 mg/kg did not sensitize mice to endotoxin. Sensitization by pyran required about 24 hr. The LD_{50} for endotoxin was greater than 30 mg/kg when administered 4 and 8 hr after pyran. However, sensitization to endotoxin was present as seen in an LD_{50} of 0.3 mg/kg 24 hr post pyran (Table III).

TABLE II. Survival Times of Control and Pyran-Treated Mice Receiving Lethal Numbers of *D. pneumoniae* and *S. aureus*.[a]

	Survival time (days)	
	D. pneumoniae	*S. aureus*
Control	3	2
Pyran copolymer[b]	14	14

[a] Ten mice/group.

[b] Pyran administered in a dose of 25 mg/kg daily for 2 days. Microorganisms administered iv 7 days after the last drug injection.

TABLE III. Duration of Time Required for Pyran to Sensitize to Endotoxin.

Endotoxin injected after pyran[a] (hr)	LD_{50} (mg/kg)
0	>30
4	>30
8	>30
24	0.3

[a] Pyran administered in a dose of 25 mg/kg iv.

Pyran-treated mice have a decreased ability to metabolize hexobarbital (5) and antipyrine (Table IV). Twenty-four hr after the last injection of pyran there was a 200% increase in the duration of anesthesia. There was a 72% decrease in the *in vitro* metabolism of hexobarbital and a 38% decrease in antipyrine metabolism by the 9000*g* liver supernatant fraction.

To determine if the depression of microsomal enzyme activity was related to endotoxin sensitivity, pyran-treated mice received chlorcyclizine in a protocol known to enhance microsomal enzyme activity (9). Table V shows that chlorcyclizine pretreatment

TABLE IV. Inhibition of Drug Metabolism Induced by Pyran Copolymer.[a]

	Hexobarbital sleeping time[b,c]		Metabolized (μmoles/30 min)	
	Day 1	Day 7	Hexobarbital[d]	Antipyrine[e]
Control	34.4 ± 2.4	34.1 ± 3.3	0.55	1.21
Pyran	103.4 ± 12.2	65.0 ± 6.7	0.15	0.75

[a] Pyran administered in a dose of 25 mg/kg iv daily for 2 days.

[b] Mean ± SEM derived from 10 mice/group.

[c] Hexobarbital sleeping time determined 24 hr and 7 days after the last injection of pyran.

[d] Incubation vial contained 2 ml of liver supernatant fraction (100 mg of protein), 1 μmole of hexobarbital, 50 μmoles of nicotinamide, 25 μmoles of $MgCl_2$, 0.26 μmoles of DPN, and 0.1 *M* phosphate buffer (pH 7.4) to final volume of 4 ml.

[e] Same as (*d*) except 10 μmoles of antipyrine were added.

decreased the duration of anesthesia 81% while pyran alone increased the sleeping time 84%. Chlorcyclizine administered concomitantly with pyran not only reversed pyran action but decreased the sleeping time 62% over control. However, chlorcyclizine did not reverse the sensitization to endotoxin.

It has been previously shown by Thomas *et al.* (10) that heparinization aborts the generalized Shwartzman phenomena and also prevents the endotoxin-lethality in polyanetholsulfonate-treated mice. To compare pyran to polyanetholsulfonate, we heparinized mice after pyran and before endotoxin. The dose of heparin was sufficient to maintain the blood incoagulable for at least 10 hr. While heparin protected the mice from a bilateral hemorrhage of the kidneys, it only slightly prolonged the survival time, *i.e.*, from 10 to 20 hr (Table VI).

TABLE V. The inability of a Microsomal Enzyme Stimulant to Reverse the Sensitivity of Pyran Copolymer-Treated Mice to Endotoxin.

	Hexobarbital sleeping time[a] (min)	Endotoxin[e] LD_{50} (mg/kg)
Control	33.2 ± 2.1	45
Chlorcyclizine[b] 25 mg/kg iv 3	6.3 ± 0.3	45
Pyran copolymer[c] 25 mg/kg iv 2	93.2 ± 4.7	0.4
Chlorcyclizine[d] 25 mg/kg ip 3 pyran 25 mg/kg iv 2	12.7 ± 0.7	0.5

[a] Mean ± SE derived from 10 mice/group.

[b] Chlorcyclizine administered in a dose of 25 mg/kg ip daily for 3 days.

[c] Pyran copolymer administered in a dose of 25 mg/kg iv daily for 2 days.

[d] Chlorcyclizine administered in a dose of 25 mg/kg ip daily for 3 days and on the last 2 days pyran copolymer administered in a dose of 25 mg/kg iv.

[e] Endotoxin (*S. typhosa*) administered iv 24 hr after the last injection of drug.

TABLE VI. Effect of Pretreatment with Heparin on the Sensitivity of Pyran-Treated Mice to Endotoxin.

	No. dead/ no. in group	Survival time (hr)
Pyran copolymer[a]	0/10	
Pyran copolymer[b] endotoxin	10/10	10
Pyran copolymer heparin endotoxin[c]	10/10	20

[a] Pyran administered in a dose of 25 mg/kg iv.

[b] Endotoxin administered in a dose of 1 mg/kg, 24 hr after the pyran.

[c] Heparin administered in a dose of 2000 units/kg sc, 23 hr after pyran and 1 hr prior to endotoxin.

Discussion. Numerous studies have demonstrated the enhanced endotoxin sensitivity of animals pretreated with various RES active drugs and diseases involving RE organs (11–13). Early studies showed that blockade of the RES, by such agents as Thorotrast, trypan blue (14, 15), saccharide iron oxide (16), is associated with increased sensitivity. Other investigators using the simple lipids cholesterol oleate (11) and methyl palmitate

(13), indicated that RES blockade is not associated with increased sensitivity to endotoxin but provides protection from its lethal effects. In addition, endotoxin given in small doses over a period of 4–5 days produces a RES stimulation and a tolerance to endotoxin. However, endotoxin given iv also produces a RES stimulation 24 hr later, and if it is reinjected at a sublethal dose, produces a generalized Shwartzman phenomena (17). Stimulation of RE function by glucan, triolein, *Mycobacterium bovis* strain BCG, bacterial and viral infections also sensitizes animals to endotoxin (11–13).

In the present studies, pyran produces a marked biphasic response to RES activity, and the mice are sensitive to endotoxin throughout both phases. Although the RE organs normally take up most of the intravenously injected endotoxin (18, 19), it appears from these and earlier studies that the phagocytic status of the liver and spleen is not the determining factor in the sensitization to the lethal action of endotoxin.

The sensitization by pyran requires about 24 hr. If large sublethal doses for control mice are administered to pyran-treated mice during the first 8 hr, there is no increased toxicity. Previous studies from this laboratory on the fate and distribution of ^{14}C pyran show that it requires 24 hr for clearance from the vascular system (20). Also, the blockade of hepatic phagocytosis by pyran and its effect on drug metabolism requires 24 hr. This delay in the manifestation of biological activities may be related to the time necessary for pyran to reach the site of action.

The increased endotoxin sensitivity produced by pyran is not the same as that caused by the acidic polymers, polyanetholsulfonate, dextran sulfate and polyvinyl alcohol (10), or lead acetate (21). These agents must be given simultaneously with or within a few hours after endotoxin. In contrast, pyran must be given at least 24 hr prior to the endotoxin. This time interval is also necessary for pyran action on the microsomal enzymes. In this regard, the sensitivity to endotoxin produced by pyran is similar to the time schedule used to produce the generalized Shwartzman reaction with endotoxin. However, there is one difference related to the duration of sensitization: the pyran sensitization period lasts at least 7 days while the effect of the preparing dose of endotoxin does not exceed 48 hr (17). Heparinization can abort the general Shwartzman phenomena produced by the acidic polymers or endotoxin presumably by preventing intravascular coagulation (10). This is not the case for pyran. Heparinization did prevent the hemorrhagic syndrome in the kidney but did not prevent the ultimate lethal effects of endotoxin.

As previously stated, pyran depresses the microsomal oxidase enzymes which metabolize hexobarbital and antipyrine. This altered drug metabolism occurs throughout both phases of RES activity. We have previously shown that most agents which depress or stimulate the hepatic phagocytic activity depress barbiturate metabolism. Included in this group are: endotoxin, zymosan, methyl palmitate, and diethylstilbestrol (22). Pyran depression of drug metabolism can be separated from the RES activity by using SKF525A phenobarbital or chlorcyclizine. When these agents are administered to pyran-treated mice in a protocol known to enhance drug metabolism, they reverse pyran drug metabolism action but do not alter pyran action on RES function (5). If the inhibition of this enzyme system is responsible for the increased endotoxin sensitivity, we would expect to reverse pyran sensitization with chlorcyclizine treatment. As shown in Table VI, this is not the case. Chlorcyclizine reversed the drug metabolism action but did not significantly alter the sensitivity to endotoxin.

However, we cannot rule out microsomal or soluble enzymes as the site of action because they include many different enzyme systems. Skarnes (23) has shown that plasma esterase levels in endotoxin-sensitive animals are decreased. This may be a reflection of either a decreased synthesis by the liver or an inhibition of preformed esterases. Berry and co-workers (24) demonstrated a decrease in liver tryptophan pyrrolase activity after administration of endotoxin, zymosan, or glucan. Filkins has shown that lysosomal enzymes

may be involved (25). Possibly RE trophic agents or diseases bring about the production of an inhibitor of the synthesis or action of the enzyme(s) involved in the metabolism of endotoxin.

Summary. Pyran copolymer markedly sensitizes mice to the lethal effects of endotoxin. The sensitization occurs throughout both phases of RES activity. Pyran action on microsomal enzymes necessary for the metabolism of hexobarbital or antipyrine is not responsible for the sensitization because reversal of this activity with chlorcyclizine does not alter endotoxin sensitization. Preheparinization of pyran-treated mice only slightly prolonged the survival time, but did prevent the kidney damage.

The author acknowledges the technical assistance of Mrs. Judith Munson.

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Received Jan. 26, 1971. P.S.E.B.M., 1971, Vol. 137.