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Effect of Zymosan on Hepatic Drug Metabolism in Mice[1)]

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Effect of zymosan, a typical reticuloendotherial stimulator, on hepatic drug-metabolizing enzymes was examined in mice.

1) When given *in vivo*, it depressed all the activities of aminopyrine N-demethylase, *p*-nitroanisole O-demethylase, and aniline hydroxylase. It was shown that the effect of zymosan on drug metabolism was exerted solely on microsomes and there was a marked decrease of cytochrome P-450 content in microsomes of zymosan-treated animals.

2) Aminopyrine N-demethylase activity was found to become minimum 24 hours after the administration of zymosan. On the other hand, phagocytic activity was enhanced at the same time when the maximum depression of the metabolism of aminopyrine was observed.

It is well known that the activity of hepatic drug-metabolizing enzyme could be altered by various physiological conditions as well as by the administration of various drugs. Some investigators[3)] have reported the effects of modifiers of reticuloendotherial (RE) activities on the drug metabolism. In the early days Samaras and Dietz[3a)] had reported that barbiturate-induced sleeping time was prolonged during RE blockade. On the contrary it had been shown that agents which stimulate the functional activity of the RE system also prolonged the duration of hexobarbital anesthesia.[3b,3c,3d)]

Recently Wooles and Munson[4)] investigated precisely the effect of RE stimulator and depressant on drug metabolism attempting to explain above mentioned discrepancy. Consequently, they speculated that regardless of the functional state of the RE system produced drugs which alter phagocytosis depress the metabolism of barbiturates. However, the mechanism of the effect of RE modifiers on drug metabolism has not been so far fully elucidated.

In the present study we deal with the effect of zymosan, one of the RE stimulants, on hepatic drug-metabolizing enzyme activities, the content of cytochrome P-450, and other biological parameters.

Materials and Methods

Materials——Zymosan type A, glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP), and its reduced type (NADPH) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Carbon suspension, C11-1431a, was purchased from Gunter Wagner (Hannover, Germany).

Treatment of Animals——Male ddY strain of mice weighing 21—26 g were used and 40 mg/kg of zymosan suspended in 0.9% NaCl solution was injected intraperitoneally (*i.p.*) twice at five hours interval for most of the present experiments. As the control 0.9% NaCl solution was given. Animals were sacrificed for the assay of drug-metabolizing enzyme activities between 10: 30—11: 30 AM. to minimize a daily rhythmic variation.

1) A part of this work was presented at the 14th Meeting of Tohoku Branch, Pharmaceutical Society of Japan, Sendai, October, 1974.

2) Location: *a*) *Aobayama, Sendai*; *b*) *1–18–1, Kamiyoga, Setagaya-ku, Tokyo.*

3) *a*) S.C. Samaras and N. Dietz, Jr., *Federation Proc.*, **12**, 122 (1953); *b*) W.R. Wooles and J.F. Borzelleca, *J. Reticuloendothel. Soc.*, **1**, 354 (1964); *c*) F.J. DiCarlo, L.J. Haynes, C.B. Countiho, and G.F. Phillips, *J. Reticuloendothel. Soc.*, **2**, 360 (1965); *d*) D.W. Barnes and W.R. Wooles, *J. Reticuloendothel. Soc.*, **7**, 684 (1969).

4) W.R. Wooles and A.E. Munson, *J. Reticuloendothel. Soc.*, **9**, 108 (1971).

Preparation of 9000×*g* Supernatant and Microsomal Fractions——The livers removed after decaptation were rinsed, and thoroughly perfused in cold 0.9% NaCl solution for the convenience to the assay of cytochrome P-450, and then homogenized with 4 vol. of cold 1.15% KCl solution using a Potter-Elvehjem teflon-glass homogenizer. The liver homogenates obtained were centrifuged at 9000×*g* for 30 min. The resultant supernatant fractions were centrifuged at 105000×*g* for 60 min to obtain microsomal pellets using a Hitachi 40p preparative ultracentrifuge. The microsomal pellets were resuspended in isotonic KCl solution with a homogenizer. For the assay of cytochrome P-450 content, microsomes were washed again as mentioned above and finally suspended in 0.1 M phosphate buffer (pH 7.4) at a concentration of 2 mg of protein per ml.

Assay of Drug-Metabolizing Enzymes and the Contents of Cytochrome P-450 and Protein——The incubation mixture for the assay of drug-metabolizing enzymes consisted of 2.0 ml of the 9000×*g* supernatant fraction (35 mg of protein), 1.0 ml of 0.1 M phosphate buffer (pH 7.4), 50 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 0.27 μmole of NADP, 1.92 μmoles of G-6-P, and 5 μmoles of substrate in a final volume of 5.0 ml. Incubation was conducted at 37° for 20 min. When microsomal fraction was used as the enzyme source 0.5 unit of G-6-P dehydrogenase was added to the incubation medium. The enzyme activities were calculated from the amount of product yielded by the reaction. Formaldehyde produced by the demethylation of aminopyrine was determined by the method of Cochin and Axelrod.[5] *p*-Nitrophenol produced from *p*-nitroanisole was determined by the method of Netter and Siedel.[6] *p*-Aminophenol produced from aniline was determined by the method of Imai and Sato.[7]

Determination of cytochrome P-450 were performed by the method of Omura and Sato.[8] Protein content was assayed by the method of Lowry, *et al.*[9]

Assay of Phagocytic Activity——The phagocytic activity was evaluated by measuring the intravascular removal rate of colloidal carbon according to the description by Biozzi, *et al.*[10] Colloidal carbon (16 mg/100 g body weight) was injected to the tail vein of mice. The blood samples were taken up from the retro-orbital venous plexus in mice at 2 and 12 min after the injection. The phagocytic index, K, was calculated from the following equation:

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

where C_1 and C_2 are the carbon concentrations at time T_1 and T_2 and K is a measure of the rate of phagocytosis of carbon particles by the RE system for a given dose of carbon.

Results

Effect of Zymosan Administration on Some Drug-Metabolizing Enzymes

Mice of ddY strain were treated with double injections of 40 mg/kg of zymosan at five hours interval. The drug-metabolizing enzyme activities of 9000×*g* supernatant fractions from liver homogenates were examined 24 hours after the first injection (Table I). Aminopyrine N-demethylation, *p*-nitroanisole O-demethylation, and aniline hydroxylation were all depressed about 40% compared to control. Sex differences were not observed in the effect of zymosan. The intravenous administration of zymosan resulted in a little more inhibitory action on aminopyrine N-demethylation than intraperitoneal application, but mice rarely died with the embolism in capillarys by the intravenous injection. Therefore, zymosan was given intraperitoneally in the following studies.

Changes in Aminopyrine N-Demethylase Activity and Phagocytosis after Zymosan Administration

Phagocytic activities were measured by the carbon clearance method. It is known that 90% of colloidal carbon injected is incorporated under normal conditions in the liver. Table II shows the responses of aminopyrine N-demethylating activity and phagocytic activity to the dosing of zymosan. Both responses depended on the dose of zymosan. At the dose of 10

5) J. Cochin and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **125**, 105 (1959).
6) K.J. Netter and G. Siedel, *J. Pharmacol. Exptl. Therap.*, **146**, 61 (1964).
7) J. Imai and R. Sato, *J. Biochem.* (Tokyo), **60**, 417 (1966).
8) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2370 (1964).
9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) G. Biozzi, B. Benacerraf, and B.N. Halpern, *Brit. J. Exptl. Pathol.*, **34**, 441 (1953).

Table I. Effect of Zymosan Administration on Some Microsomal Drug-Metabolizing Enzyme Activities

Substrate	Route of injection	Enzyme activity		Per cent of control
		Control	Zymosan	
		Males		
Aminopyrine	*i.p.*	3.19±0.32	2.02±0.41[a]	63.3
Aminopyrine	*i.v.*	3.53±0.39	1.85±0.13[a]	52.4
Aniline	*i.p.*	1.10±0.10	0.70±0.19[a]	63.6
p-Nitroanisole	*i.p.*	1.39±0.10	0.83±0.10[a]	59.7
		Females		
Aminopyrine	*i.p.*	2.79±0.23	1.94±0.08[a]	69.5
Aniline	*i.p.*	1.13±0.09	0.73±0.07[a]	64.6

Mice were injected with zymosan (40 mg/kg) twice at five hours interval. The enzyme activities were assayed 24 hr after the first injection and represented by the mean ±S.D. of metabolites produced in μmoles per g liver per hr. Groups of 6 animals were used, the activity being estimated in duplicate on 3 paired 9000×*g* supernatant fractions.
a) significantly different from control, $p<0.01$

Table II. Effect of Amount of Zymosan on the Metabolism of Aminopyrine and Phagocytosis

Dose (mg/kg)	Aminopyrine N-demethylase[a]	Phagocytosis[b] ($K\times10^2$)
Control	2.92±0.04	2.53±0.46
Zymosan		
1	2.80±0.06	2.52±0.44
10	2.27±0.03[c]	3.93±0.73[c]
40	1.78±0.02[c]	4.10±0.64[c]
80	1.69±0.04[c]	3.19±0.76

Male mice were injected *i.p.* with zymosan twice at five hours interval. The enzyme activity and the phagocytosis were measured 24 hr after the first injection.
a) expressed as the mean ±S.D. of the values (μmoles HCHO/g liver/hr) obtained with 12 animals, the activity being determined on 6 paired liver preparations, *b*) expressed as the mean ±S.D. of the values ($K\times10^2$) obtained with 9 animals, *c*) significantly different from control, $p<0.01$

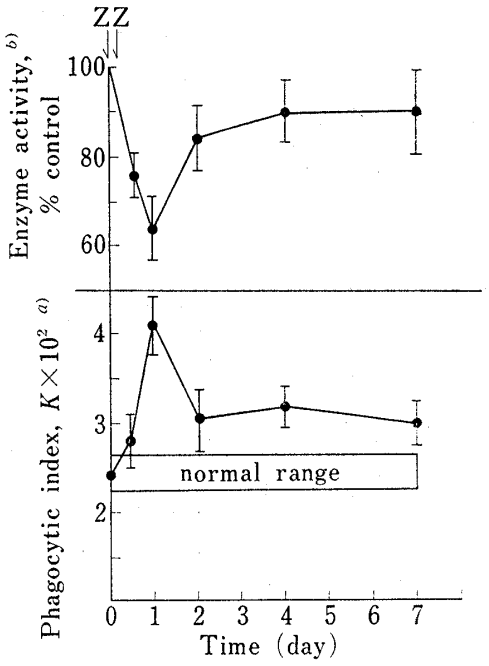

Fig. 1. Time Courses of the Metabolism of Aminopyrine and Phagocytosis after *i.p.* Administration of Zymosan

Mice were injected with zymosan (40 mg/kg) twice at five hours interval.
a) expressed as the mean ±S.D. of the values obtained with 12 animals, the activity being determined on 6 paired liver preparations
b) expressed as the mean ± S.D. of the values obtained with 9 animals

mg/kg of zymosan the metabolism of aminopyrine was depressed and the phagocytic activity was stimulated, while by 1 mg/kg the both activities were not changed. Fig. 1 shows the changes of above mentioned two activities at various time intervals after the first administration of zymosan. N-Demethylase activity was found to become minimum after 24 hours and then the activity rebounded gradually to the normal level. On the other hand, enhanced phagocytic action took place at the same time when the maximum depression of aminopyrine demethylation was observed. It appears that these two activities responded oppositely each other to an administration of zymosan at least in the early stage.

Effect of Zymosan on the Drug-Metabolizing Enzyme Activities, the Content of Cytochrome P-450 and Protein in Hepatic Microsomal Fraction

It was known that the microsomal drug-metabolizing enzyme activities are affected by the concentrations of cofactors[11] and the activating factor(s)[12] in cell sap. It was felt that changes of these factors might bring about the depression observed above.

Therefore, the recombination experiment was carried out with microsomal and 105000×***g*** supernatant fractions prepared from control and treated animals (Table III). Activity of N-demethylase was not changed when the 105000×***g*** supernatant fraction from control animal was exchanged with that from treated animal. The depression of the enzyme activity was reproduced when the microsomes from the treated animal were employed.

Table III. Recombination of Hepatic Subfractions Obtained from Control and Zymosan-Treated Animals

Recombination		Aminopyrine N-demethylase	Per cent of control
Microsomes	Supernatant		
Control	control	0.172±0.018	100
Control	zymosan	0.172±0.003	100.0
Zymosan	control	0.127±0.005	73.8
Zymosan	zymosan	0.129±0.009	75.0

Mice were treated with zymosan (40 mg/kg) twice at five hours interval. 24 hr after the first injection, microsomal and 105000×***g*** supernatant fractions were prepared from control and zymosan-treated animals. The cellular fractions were recombined as indicated. Results were expressed as the mean ± S.D. of the metabolites produced in μmoles per mg of microsomal protein per hr. 12 animals were used in each group.

Besides the drug-metabolizing enzyme activity the contents of cytochrome P-450 and proteins in microsomes and 105000×***g*** supernatant were measured as well as the weights of body, liver, and spleen (Table IV). Although the stimulation of phagocytosis was resulted by zymosan treatment, hypertrophies of the liver and spleen were not observed. The ratios of these organ weights against body weight were not changed by zymosan treatment, while the body weight itself was slightly but significantly decreased in the treated animals. Protein contents in 105000×***g*** supernatant fractions were unchanged and those of microsomes were depressed 12.8%. The content of cytochrome P-450 per g liver in treated animals decreased 42% and even on a protein basis it decreased 29% compared to control animals. Since cytochrome P-450 plays an important and essential role in the drug-metabolizing enzyme system, above result reveals that the decrease of cytochrome P-450 content is closely influencing on the depression of drug-metabolizing activity in zymosan-treated mice.

Effect of Zymosan on Microsomal Drug Metabolism and Cytochrome P-450 in Fasting Condition

Following the double injections of zymosan the food intake of treated animals decreased 35—40% in comparison with controls. Since drug-metabolizing enzyme activities are usually affected by food intake, the effects of zymosan were investigated using the fasted mice which were starved from the time when the first dose of zymosan was given.

Although the fasting itself brought about 28% depression of aminopyrine N-demethylation and, on the contrary, 34% acceleration of aniline hydroxylation, zymosan treatment suppressed both drug-metabolizing reactions regardless of substrates as was observed with fed animals. Cytochrome P-450 content which was not affected by fasting was decreased

11) K.J. Netter, *Arch. Exptl. Pathol. Pharmakol.*, **238**, 292 (1960).

12) *a*) R.A. Van Dyke and C.G. Wineman, *Biochem. Pharmacol.*, **20**, 463 (1971); *b*) C. Ikeda and F. Wada, *Seikagaku (Tokyo)*, **45**, 234 (1973); *c*) T. Kamataki, N. Ozawa, M. Kitada, and H. Kitagawa, *Biochem. Pharmacol.*, **23**, 2485 (1974).

Table IV. Effect of Zymosan on the Content of Cytochrome P-450 and Protein in Hepatic Subfractions and the Weight of Liver and Spleen

	Control	Zymosan	% control
Body weight, g	24.0±0.5	22.7±0.5[a]	94.6
Liver, % body weight	5.94±0.27	5.90±0.26	99.3
Spleen, % body weight	0.52±0.10	0.58±0.12	111.5
Protein, mg/g liver			
9000× supernatant	86.7±5.0	81.5±3.6	94.0
105000×*g* supernatant	69.5±1.8	67.8±1.3	97.6
Microsomes	17.2±0.6	15.0±1.2[a]	87.2
Cytochrome P-450			
nmoles/mg protein	1.12±0.08	0.79±0.04[b]	70.5
nmoles/g liver	19.2	11.2	58.3

Mice were treated with zymosan (40 mg/kg) twice at five hours interval and killed 24 hr after the first injection. Cytochrome P-450 content per g liver was calculated from the amount of microsomal protein and the specific content of cytochrome P-450 in microsomes. Results were expressed as the mean ±S.D. of the values obtained with 18 animals.
a) significantly different from control, $p<0.05$,
b) significantly different from control, $p<0.01$

Table V. Effect of Zymosan on Drug-Metabolizing Enzymes and Cytochrome P-450 in Hepatic Microsomes of Fasting Mice

Animals	Treatment	Aminopyrine N-demethylase	Aniline hydroxylase	Cytochrome P-450
Fasting	saline	0.109±0.019	0.095±0.009	1.09±0.06
	zymosan	0.067±0.011[a]	0.074±0.003[b]	0.83±0.05[b]
	% control	61.5	77.9	76.1
Fed	saline	0.151±0.009	0.071±0.015	
	zymosan	0.099±0.012[a]	0.051±0.006[b]	
	% control	65.6	71.8	

Mice were treated with zymosan (40 mg/kg) twice at five hours interval and killed 24 hr after the first injection. Starvation was started 24 hr before the animals were killed. Enzyme activities were expressed as the mean ± S.D. of the metabolites produced in μmoles per mg of microsomal protein per hr and cytochrome P-450 content was expressed as the mean ±S.D. of the concentrations in nmoles per mg of microsomal protein. Groups of 12 animals were used in each experiment.
a) significantly different from control, $p<0.05$, *b*) significantly different from control, $p<0.01$

by the administration of zymosan. Therefore, the decrease of food intake could be excluded from the possible reasons for the depression of drug metabolism produced by zymosan treatment.

Discussion

Administration of zymosan, a typical stimulator of the RE system, brought about the depression of hepatic drug-metabolizing enzyme activities in mice. Such effect was observed in all cases of the metabolism of both type-I and type-II substrates. The facts that the effect of zymosan was exerted solely on the microsomes and not on the supernatant fractions and there was a marked decrease of cytochrome P-450 in hepatic microsomes from zymosan-treated animals reveals that the decrease of cytochrome P-450 content may be the main cause for the depression of drug metabolism.

Recently, Gaillard, *et al.*[13] reported that various lipid-emulsions, inhibitors of the RE system, decrease the enzymatic activities of aniline aromatic hydroxylase and *p*-nitroanisole

13) D. Gaillard, B. Pipy, and R. Derache, *Biochem. Pharmacol.*, 23, 1245 (1974).

O-demethylase, but have no effect on the N-demethylation. In their experiment the amount of cytochrome P-450 was not changed. Even if drugs which alter phagocytosis depress the metabolism of drugs, the present study suggested that the inhibitory mechanisms of drug metabolism by the stimulators of the RE system such as zymosan might be different from the inhibitory mechanisms caused by lipid-emulsions as mentioned above at least in respect of the alteration of cytochrome P-450 contents.

However, the present data did not establish whether the decrease of cytochrome P-450 content was an accompanying event to a zymosan-induced stimulation of phagocytic activity or was a separate effect of the agent not related to its ability to stimulate phagocytosis.