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## Different Depressing Effects of Lentinan on the Increases of Cytochrome P-450-Dependent Monooxygenase Activities Induced in Mice by Phenobarbital and by 3-Methylcholanthrene

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The effect of lentinan (1 mg/kg/12 h  $\times$  4, *i.p.*) on the hepatic cytochrome P-450 molecular species and the cytochrome P-450-dependent monooxygenase activities was observed in ddY and C57BL/6 mice treated with phenobarbital (8 or 40 mg/kg/d  $\times$  2, *i.p.*) or 3-methylcholanthrene (80 mg/kg, *i.p.*). The activities of aminopyrine *N*-demethylase, aniline hydroxylase and biphenyl 4-hydroxylase were elevated by 20—37% by the treatment with phenobarbital, and these increases were unaffected by concurrent administration of phenobarbital and lentinan. The activities of 7-ethoxycoumarin *O*-deethylase and biphenyl 2-hydroxylase were increased by 74—192% by the treatment with 3-methylcholanthrene, but these increases were depressed by the combination treatment with 3-methylcholanthrene and lentinan. It was found by an sodium dodecyl sulfate-polyacrylamide gel electrophoretic study that the suppression by lentinan of the elevation of cytochrome P-450-dependent monooxygenase activities induced by 3-methylcholanthrene was caused by a decrease in the hepatic microsomal cytochrome P-450 content.

**Keywords**—lentinan; phenobarbital; 3-methylcholanthrene; aminopyrine *N*-demethylase; aniline hydroxylase; 7-ethoxycoumarin *O*-deethylase; biphenyl 2-hydroxylase; biphenyl 4-hydroxylase; mouse

It has been reported that lentinan,  $\beta$ -(1—3)-glucan, shows antitumor, phagocytic and adjuvant activities in mice, as well as causing an increase in serum protein.<sup>1—3)</sup> In the previous papers, we reported that lentinan reduced the cytochrome P-450 content and the hepatic drug-metabolizing enzyme activities when aminopyrine, aniline, 7-ethoxycoumarin and biphenyl were used as substrates.<sup>4,5)</sup> In recent years, the heterogeneity of hepatic microsomal cytochrome P-450 has been demonstrated, with the progress of methods for purification.<sup>6,7)</sup> The existence of many distinct forms of cytochrome P-450 has become apparent, based on studies of the spectral properties, immunological properties, molecular weight (determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis), peptide mapping, substrate specificity and amino acid sequence.<sup>8)</sup> Nebert *et al.*<sup>9)</sup> reported that the process of induction of cytochrome P-450 by treatment with a polycyclic hydrocarbon such as 3-methylcholanthrene or benzo[*a*]pyrene differs from that with chemicals such as phenobarbital.

In the present paper, the effect of lentinan on the increase of hepatic cytochrome P-450-dependent monooxygenase activities induced by phenobarbital or 3-methylcholanthrene in mice was investigated by measuring the changes of hepatic drug-metabolizing enzyme activities and by SDS-polyacrylamide gel electrophoretic analysis.

### Experimental

**Animals**—Male ddY and C57BL/6 mice (22—24 g) were purchased from Shizuoka Laboratory Animal Center. Animals were allowed to acclimatize for at least 3 d before use in experiments and were fed water and a standard diet

(CE-2, Clea Japan, Inc.). Unless otherwise stated, groups of 6–12 mice were used in the experiments. Lentinan (1 mg/kg, *i.p.*) was given every 12 h for 2 d and mice were sacrificed 12 h after the last administration of lentinan. Phenobarbital (8 or 40 mg/kg, *i.p.*) was given every day for 2 d and mice were sacrificed 24 h after the last administration of phenobarbital. A single dose of 3-methylcholanthrene (80 mg/kg, *i.p.*) was given 48 h before the enzyme assay.

**Chemicals**—Lentinan was supplied by Morishita Pharmaceutical Co., Ltd. Reduced nicotinamide adenine dinucleotide (NADH), glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma. Glucose-6-phosphate and 7-ethoxycoumarin were purchased from Nakarai Chemical Co., Ltd. Aniline and aminopyrine were purchased from Wako Chemical Co., Ltd. and Yamada Pharmaceutical Co., Ltd., respectively. Other reagents used were of analytical grade.

**Analytical and Assay Methods**—Cytochrome P-450 content was determined by the method of Omura and Sato.<sup>10)</sup> Aminopyrine *N*-demethylase and aniline hydroxylase activities were determined by the methods of Nash<sup>11)</sup> and Imai *et al.*,<sup>12)</sup> respectively. 7-Ethoxycoumarin *O*-deethylase and biphenyl 2- or 4-hydroxylase activities were measured by the methods of Jacobson *et al.*<sup>13)</sup> and Creaven *et al.*,<sup>14)</sup> respectively. Protein was determined by the method of Lowry *et al.*<sup>15)</sup> SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli<sup>16)</sup> and densitometric scanning was performed at 550 nm using a Shimadzu CS-910 chromatoscanner.

## Results

### Effect of Lentinan on the Increase in Hepatic Cytochrome P-450 Content and Drug-Metabolizing Enzyme Activities Induced by Phenobarbital

Cytochrome P-450 content and the activities of aminopyrine *N*-demethylase, aniline hydroxylase and biphenyl 4-hydroxylase were increased significantly by the treatment with phenobarbital (8 mg/kg/d  $\times$  2, *i.p.*), the extents of increase being 15, 30, 20 and 37%, respectively, compared to the control values. When phenobarbital and lentinan (1 mg/kg/12 h  $\times$  4, *i.p.*) were used together, no effect was observed on the above parameters in comparison with those in mice treated with phenobarbital alone.

### Relation between the Dose of Lentinan and the Increase of Hepatic Aminopyrine *N*-Demethylase Activity Induced by Phenobarbital

The activity of aminopyrine *N*-demethylase was induced by the treatment with phenobarbital (8 mg/kg/d  $\times$  2, *i.p.*) alone. When phenobarbital and lentinan (1, 2 or 10 mg/kg/12 h  $\times$  4, *i.p.*) were used together, lentinan did not affect the increase of aminopyrine *N*-demethylase activity induced by phenobarbital.

### Effect of Lentinan on the Increase in Hepatic Cytochrome P-450 Content and Drug-Metabolizing Enzyme Activities Induced by 3-Methylcholanthrene

Cytochrome P-450 content and the activities of 7-ethoxycoumarin *O*-deethylase and biphenyl 2-hydroxylase were increased by treatment with 3-methylcholanthrene (80 mg/kg,

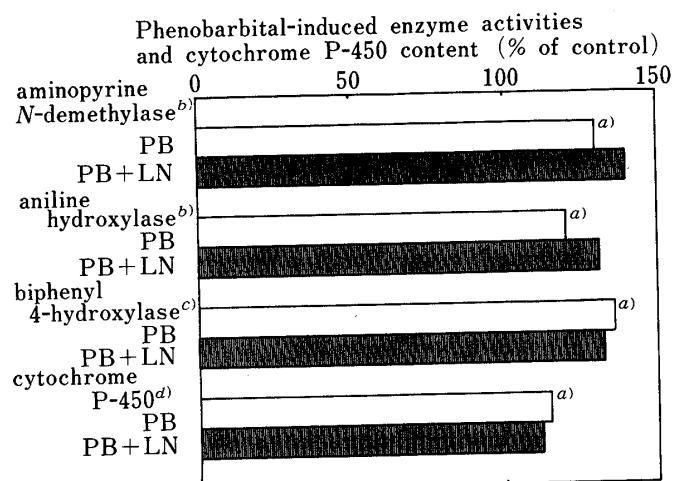


Fig. 1. Effect of Lentinan on the Increase in Hepatic Cytochrome P-450 Content and Drug-Metabolizing Enzyme Activities Induced by Phenobarbital

Eight male ddY mice were injected with 8 mg/kg of phenobarbital each day for 2 d, and sacrificed 24 h after the last administration. Lentinan (1 mg/kg, *i.p.*) was given every 12 h for 2 d and the mice were sacrificed 12 h after the last administration. Control mice were similarly treated with physiological saline. Control values: aminopyrine *N*-demethylase,  $160.2 \pm 11.14$ ; aniline hydroxylase,  $72.8 \pm 4.13$ ; biphenyl 4-hydroxylase,  $1.02 \pm 0.092$ ; cytochrome P-450,  $1.02 \pm 0.029$ . Significant differences from the control values are indicated as a) ( $p < 0.05$ ). Abbreviations: PB = phenobarbital, LN = lentinan. b) nmol/20 min/mg of protein. c) nmol/15 min/g of liver. d) nmol/mg of protein.

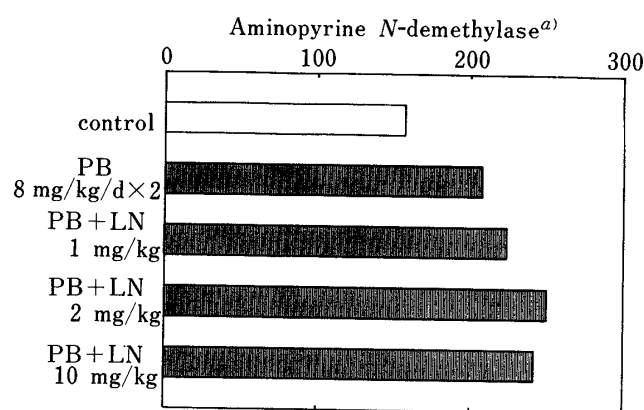


Fig. 2. Relationship between the Dose of Lentinan and the Increase of Hepatic Aminopyrine *N*-Demethylase Activity Induced by Phenobarbital

Lentinan (1, 2 or 10 mg/kg, *i.p.*) was given every 12 h for 2 d and mice were sacrificed 12 h after the last administration. The experimental conditions and method of analysis were the same as in Fig. 1. *a)* nmol/20 min/mg of protein.

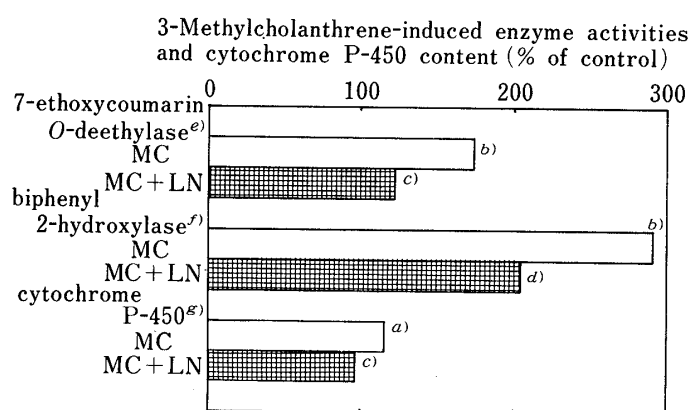


Fig. 3. Effect of Lentinan on the Increase in Hepatic Cytochrome P-450 Content and Drug-Metabolizing Enzyme Activities Induced by 3-Methylcholanthrene

Twelve male ddY mice were used. 3-Methylcholanthrene (80 mg/kg, *i.p.*) was given 48 h before the enzyme assay. Lentinan (1 mg/kg, *i.p.*) was given every 12 h for 2 d, and the mice were sacrificed 12 h after the last administration. Control values: 7-ethoxycoumarin *O*-deethylase,  $1.20 \pm 0.074$ ; biphenyl 2-hydroxylase,  $0.49 \pm 0.068$ . Significant differences are indicated as *a)* ( $p < 0.05$ ) and *b)* ( $p < 0.01$ ) vs. control value, and *c)* ( $p < 0.05$ ) and *d)* ( $p < 0.01$ ) vs. value of mice treated with 3-methylcholanthrene. Abbreviations: MC = 3-methylcholanthrene, LN = lentinan. *e)* nmol/15 min/mg of protein. *f)* nmol/15 min/g of liver. *g)* nmol/mg of protein.

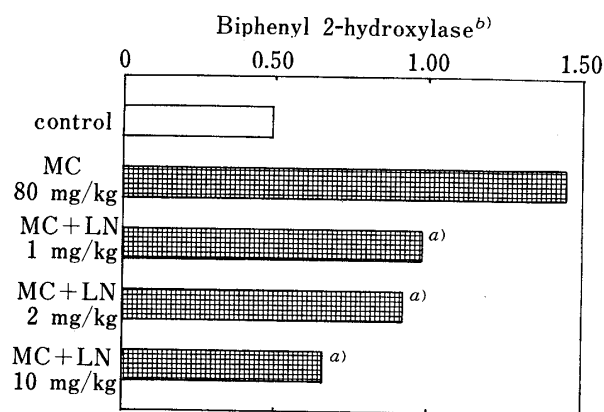


Fig. 4. Relationship between the Dose of Lentinan and the Increase of Hepatic Biphenyl 2-Hydroxylase Activity Induced by 3-Methylcholanthrene

A single dose of 3-methylcholanthrene (80 mg/kg, *i.p.*) was given 48 h before enzyme assay. Lentinan (1, 2 or 10 mg/kg, *i.p.*) was given twice a day for 2 d and mice were sacrificed 12 h after the last administration. Significant differences from the value of mice treated with 3-methylcholanthrene are indicated as *a)* ( $p < 0.01$ ). The experimental conditions and method of analysis were the same as in Fig. 3. *b)* nmol/15 min/g of liver.

*i.p.*) and the increases were 15, 174 and 292% as compared with the control values, respectively. On the other hand, when 3-methylcholanthrene and lentinan (1 mg/kg/12 h × 4, *i.p.*) were used together, these increases of the enzyme content and activities were depressed significantly in comparison with those in mice treated with 3-methylcholanthrene alone.

**Relationship between the Dose of Lentinan and the Increase of Hepatic Biphenyl 2-Hydroxylase Activity Induced by 3-Methylcholanthrene**

The activity of biphenyl 2-hydroxylase was increased by the treatment with 3-methylcholanthrene (80 mg/kg, *i.p.*) alone. When 3-methylcholanthrene and lentinan (1, 2 or 10 mg/kg/12 h × 4, *i.p.*) were used together, however, the activity was depressed dose-dependently in comparison with that in mice treated with 3-methylcholanthrene alone.

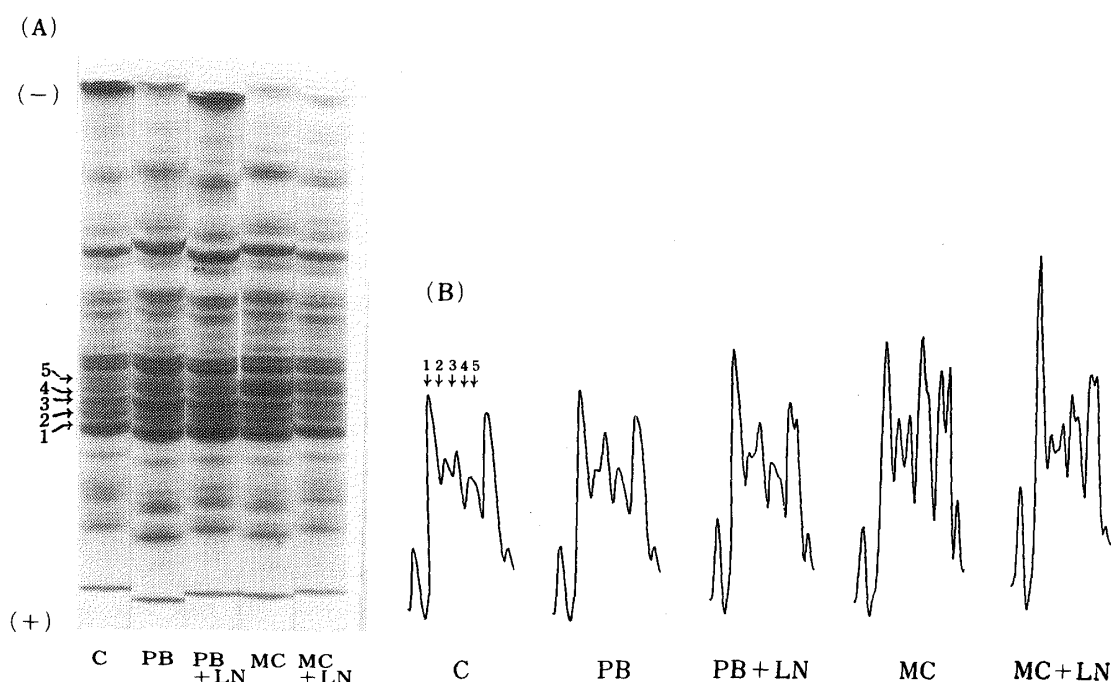


Fig. 5. Effect of Lentinan on the Hepatic Cytochrome P-450 Molecular Species Induced by Phenobarbital or 3-Methylcholanthrene, as Determined by SDS-Polyacrylamide Gel Electrophoresis

(A): A typical electrophoretic pattern of the hepatic microsomes in male C57BL/6 mice after treatment with drugs (migration from top to bottom). Polyacrylamide gel (7.5%) was used. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and destained with isopropyl alcohol/acetic acid. Each sample was applied to the gel at a dose of 20  $\mu$ g of microsomal protein.

(B): Scans of the 50000 molecular weight region of the gels shown in Fig. 5 (A) (absorbance at 550 nm). Abbreviations: C=control, PB=phenobarbital, PB+LN=phenobarbital and lentinan, MC=3-methylcholanthrene, MC+LN=3-methylcholanthrene and lentinan.

### Effect of Lentinan on the Hepatic Cytochrome P-450 Molecular Species Induced by Phenobarbital or 3-Methylcholanthrene as Judged by SDS-Polyacrylamide Gel Electrophoresis

It is apparent that the synthesis of certain cytochrome P-450 molecular species was induced by the treatment with phenobarbital (40 mg/kg/d  $\times$  2, *i.p.*) or 3-methylcholanthrene (80 mg/kg, *i.p.*) in the hepatic microsomes. Therefore, the effect of lentinan (1 mg/kg/12 h  $\times$  4, *i.p.*) on the microsomal cytochrome P-450 molecular species in C57BL/6 mice was studied by means of SDS-polyacrylamide gel electrophoresis. By staining of the gel with a protein-binding dye, it was seen that cytochrome P-450 molecular species exist as five bands in the range of molecular weight from 45000 to 60000. The molecular weights of bands 1, 2, 3, 4 and 5 were about 46000, 48000, 49000, 53000 and 54000, respectively. Band 3 was stained strongly after phenobarbital treatment. However, when phenobarbital and lentinan were used together, no effect was observed on the intensity on band 3 staining in comparison with that in the case of mice treated with phenobarbital alone. On the other hand, bands 4 and 5 were stained strongly after the treatment with 3-methylcholanthrene. However, when 3-methylcholanthrene and lentinan were used together, the staining of bands 4 and 5 was weak in comparison with that in mice treated with 3-methylcholanthrene alone. The changes of these bands can be seen in Fig. 5 (B), which is a scan of the protein banding pattern in the 50000 molecular weight region of these gels.

### Discussion

Recently, methods for the purification of cytochrome P-450 have been improved

markedly. In consequence, characteristic types of cytochrome P-450 have been discovered to be induced by treatment with differential inducers of drug-metabolizing enzymes.<sup>17)</sup> Furthermore, from *in vitro* inhibition studies it is known that metyrapone and SKF-525A inhibit the metabolism of several substrates in the hepatic microsomes of animals treated with phenobarbital.<sup>18,19)</sup> Further, 7,8-benzoflavone specifically inhibits the cytochrome P-450-dependent monooxygenase induced by 3-methylcholanthrene *in vitro*.<sup>20)</sup> However, it has not been reported that immunopotentiating agents show any specific inhibitory effect on the cytochrome P-450-dependent monooxygenase activities *in vivo*.

On the other hand, it has been reported that immunopotentiating agents reduce the hepatic cytochrome P-450-dependent drug-metabolizing enzyme activities by many mechanisms, including a decrease of hepatic heme by the inhibition of  $\delta$ -aminolevulinic acid synthetase or the induction of heme oxygenase activity, and by causing liver damage.<sup>21-23)</sup> However, it was not clear whether these immunopotentiating agents non-specifically decreased the total cytochrome P-450 or specifically decreased a particular cytochrome P-450 molecular species. That is to say, the effect of immunopotentiating agents on the cytochrome P-450 molecular species has not been investigated thoroughly up to now.

In the present work, we investigated the effect of lentinan on the hepatic microsomal cytochrome P-450 to elucidate the changes of cytochrome P-450-dependent drug-metabolizing enzyme activities and molecular species by means of SDS-polyacrylamide gel electrophoretic analysis.

The hepatic cytochrome P-450 content and the activities of aminopyrine *N*-demethylase, aniline hydroxylase and biphenyl 4-hydroxylase were increased significantly by the treatment with phenobarbital. When phenobarbital and lentinan were used together, no effect of lentinan on the increases was observed in comparison with mice treated with phenobarbital alone. On the other hand, 7-ethoxycoumarin *O*-deethylase, biphenyl 2-hydroxylase activities and cytochrome P-450 content were induced markedly by treatment with 3-methylcholanthrene. When 3-methylcholanthrene and lentinan were used together, these increases in the enzyme activities were depressed in comparison with those in mice treated with 3-methylcholanthrene alone, and the depression was dose-dependent. The changes of drug-metabolizing enzyme activities were related to the cytochrome P-450 content. Thus, lentinan rather specifically affected the cytochrome P-450 molecular species induced by 3-methylcholanthrene.

It is known that C57BL/6 mice show high sensitivity for induction of 3-methylcholanthrene-dependent drug-metabolizing enzyme activities, such as aryl hydrocarbon hydroxylase.<sup>24)</sup> Therefore, C57BL/6 mice were used in order to examine the effect of lentinan on the hepatic microsomal cytochrome P-450 molecular species induced by phenobarbital or 3-methylcholanthrene by means of SDS-polyacrylamide gel electrophoretic analysis. Lentinan did not change the staining intensity of the electrophoretic bands when administered with phenobarbital, but the intensity was decreased when lentinan and 3-methylcholanthrene were used together in comparison with that in the case of mice treated with 3-methylcholanthrene alone.

The above results indicate that lentinan specifically suppresses the hepatic drug-metabolizing enzyme activities induced by 3-methylcholanthrene by decreasing the content of the relevant cytochrome P-450 molecular species. The suppressive effect of lentinan has not been detected *in vitro* but was recognized *in vivo*.<sup>5)</sup> Thus, the effect of lentinan on the hepatic drug-metabolizing enzyme activities is probably host-mediated. We are now investigating the mechanism of this action of lentinan.

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