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Liver regeneration after carbon tetrachloride intoxication in the rat

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Carbon tetrachloride (CCl_4) is a typical hepatotoxin causing hepatocellular necrosis [1]. As a result of extensive studies, the initial event in the rat given CCl_4 has been established to be lipid peroxidation of the endoplasmic reticulum of the liver cell mediated by cytochrome P-450 [1, 2]. However, only limited information is available on the successive processes after lipid peroxidation leading to necrosis [3] as well as on the defensive responses of the rat.

In 1959, Leevy *et al.* [4] advanced histological evidence which showed that mitosis increased in the rat liver after CCl_4 intoxication. Based on this study, administration of CCl_4 was assumed to cause liver regeneration and sometimes was used as an alternative method of partial hepatectomy [5–7]. Production of α-fetoprotein [8–10], increased synthesis of polyamines [11], changes in hormone receptors [10], and expression of a cellular oncogene [6] took place in the rat liver on feeding of CCl_4. These observations were discussed in relation to the results of partial hepatectomy, and a regenerative response was suggested to arise in CCl_4 poisoned liver.

In this paper, we present direct biochemical evidence of liver regeneration after administration of CCl_4 by showing increased activities of DNA-synthesizing enzymes, especially focused on thymidylate synthetase (TS, EC 2.1.1.45) and thymidine kinase (TK, EC 2.7.1.21). TS is responsible for the *de novo* synthesis of dTMP by the methylation of dUMP, and TK catalyzes the formation of dTMP by phosphorylation of 2′-deoxyribothymidine via the "salvage pathway". Activities of these enzymes increased dramatically in the liver after partial hepatectomy from almost negligible levels, and the time course of their induction closely correlated with that of DNA synthesis [12]. Many other works have shown that these two enzymes correlated closely with the rate of DNA synthesis and cell proliferation [13]. Therefore, these enzymes are believed to be rate determining in DNA synthesis [14–16], and thus the rise of their activities constitutes a reliable biochemical indicator for tissue regeneration.

Materials and methods

Materials. (±)-L-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid according to the method of Huennekens *et al.* [17]. [5-^{3}H]dUMP (14.8 Ci/mmole) and [6-^{3}H]thymidine (26 Ci/mmole) were purchased from Amersham. All other reagents were of analytical grade.

Methods. Wistar male rats, strain st, weighing 180–250 g, were obtained from the Shizuoka Laboratory Animal Co. (Shizuoka, Japan). The CCl_4 group recieved a single dose of 0.5 ml/100 g body wt of CCl_4 in corn oil (1:1, v/v) through an intragastric tube. The phenobarbital plus CCl_4 group [the (PB + CCl_4) group] received daily intraperitoneal injection of phenobarbital at a dose level of 8 mg/100 g body wt for 3 days before the injection of CCl_4. All the animals had free access to food (commercial diet, MF pellets, Oriental Animal Foods Co., Osaka, Japan) and water.

The rats of each group were killed under ether anesthesia at 24, 48, 72 and 96 hr after CCl_4 injection. Blood was collected from etherized rats by puncturing the abdominal aorta, and the livers were perfused *in situ* with cold 0.9% NaCl. The excised liver was homogenized in 0.1 M Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose and 10 mM β-mercaptoethanol (the tissue buffer ratio was 1:3, w/v). After centrifugation at 36,000 *g* for 30 min at 4°, the supernatant fraction was used for the determination of the enzymatic activities.

Enzyme assays. The activities of plasma glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1) and glutamate-pyruvate transaminase (GPT, EC 2.6.1.2) were measured spectroscopically using the diagnostic kits from Wako Pure Chemical Industries Ltd. and are expressed as Karmen units [18, 19]. The TS activity was determined by a modification of the isotopic method of Roberts [20]. The 40 μl reaction mixture contained 50 mM Tris–HCl (pH 7.5), 5 mM HCHO, 1 mM (±)-L-tetrahydrofolic acid, 10 mM dithiothreitol, 0.5 mM dUMP (5 Ci/mole) and liver supernatant fraction. After incubation at 37° for 30 min, the reaction was terminated by the addition of 200 μl of a suspension of charcoal (100 mg/ml). One hundred microlitres of the supernatant fraction resulting from centrifugation for 1 min at 10,000 *g* was mixed with 3 ml of Scintisol EX-H (Dojindo Laboratories, Japan) for determination of radioactivity. TK was assayed by determining the conversion of [^{3}H]dThd to [^{3}H]dTMP by the binding of the latter nucleotide to DEAE-cellulose disks [21]. The 30 μl reaction system contained 5 mM [5-^{3}H]thymidine (5 Ci/mole), 10 mM ATP, 100 mM NaF, 10 mM $MgCl_2$, 0.1 M Tris–HCl buffer, pH 8.0, and the liver supernatant fraction. After incubation at 37° for 15 min, the reaction was stopped by placing the mixture in an ice bath. Aliquots of 20 μl were spotted on Whatman DE-81 paper disks, which were washed with 1 mM ammonium formate, water and three times with methanol. The dried disks were placed in counting vials and spotted with 0.3 ml of 0.2 M KCl in 1 M HCl. Radioactivity was measured in 3 ml of Scintisol EX-H in a liquid scintillation counter. The enzymatic activity was expressed as pmoles of product formed per min per mg protein at 37°. The protein concentration was determined by the method of Lowry *et al.* [22] using bovine serum albumin as standard.

Results and discussion

Table 1 showed changes in plasma GOT and GPT levels at every 24 hr after a single feeding of CCl_4 (0.5 ml/100 g body wt). The level of GOT increased as much as 84 times that of the control at 24 hr and reached a maximal level (160 times) at 48 hr. At 96 hr after CCl_4 injection, the plasma GOT value returned to the control level. The level of GPT increased maximally (100 times that of the control) after 24 hr and gradually decreased to the control level at 96 hr. Since these enzymes have been shown to reflect the degree of liver damage in CCl_4 poisoning [23], the liver was damaged extensively at 24–48 hr following CCl_4 injection.

The activities of TS and TK in the liver were unchanged at 24 hr, while the plasma GOT and GPT levels rose remarkably. At 48 hr, levels of TS and TK increased by 7.4 and 4.7 times, respectively, compared with the control value as shown in Table 1. Since it is well established that TS and TK are key enzymes in DNA synthesis and high activities are maintained in proliferating normal liver (developing and partially hepatectomized) and hepatomas [24, 25], the present results clearly show that the regenerative response takes place in the CCl_4-intoxicated rat liver. At 72 hr after CCl_4 administration, levels of TS and TK mounted to 10.5 and 5.4 times that of the respective control. These ratios are comparable to the maximal values attained in the rat liver after 48 hr of 67%-partial hepa-

Table 1. Plasma enzyme and liver thymidylate synthetic enzyme levels after CCl_4 intoxication*

Treatment	Time after CCl_4 (hr)	No. of rats	Plasma GOT (Karmen units)	Plasma GPT (Karmen units)	Liver TS (pmoles/min/mg protein)	Liver TK (pmoles/min/mg protein)
Control A		6	55 ± 3	19 ± 1	3.5 ± 0.8	18.2 ± 1.3
CCl_4	24	9	4713 ± 917†	2291 ± 412†	3.5 ± 1.7	20.9 ± 1.9
	48	9	7810 ± 2157†	998 ± 363†	29.4 ± 7.9†	85.3 ± 26.4†
	72	8	659 ± 234†	163 ± 48†	41.5 ± 10.9†	97.6 ± 25.7†
	96	7	101 ± 27	49 ± 13	16.9 ± 4.3†	18.8 ± 2.4
Control B		6	61 ± 4	23 ± 2	14.5 ± 0.2†	23.8 ± 3.8
PB + CCl_4	24	9	2804 ± 484‡	1455 ± 181‡	13.3 ± 2.9§	23.9 ± 9.6
	48	8	13065 ± 1798‡	2955 ± 456‡§	54.0 ± 13.7‡	101.3 ± 8.8‡
	72	12	1532 ± 429‡	324 ± 82‡	79.7 ± 6.9‡§	168.8 ± 18.7‡§
	96	7	98 ± 18	32 ± 6	27.3 ± 5.1	23.2 ± 3.1

* Results are expressed as mean ± S.E. The control A group received an equal volume of oil without CCl_4. The control B group received treatment similar to control A after PB pretreatment. Both control groups were killed at 72 hr after oil feeding. Treatments of each group and the assay of the enzymic activities were carried out according to the procedures described in Materials and Methods.

† Significantly different ($P < 0.05$) from control A (*t*-test).

‡ Significantly different ($P < 0.05$) from control B (*t*-test).

§ Significantly different ($P < 0.05$) from the corresponding CCl_4 group (*t*-test).

tectomy [12]. This observation indicates that the CCl_4 dose of 0.5 ml/100 g body wt caused regenerative response of the liver comparable to that seen after 67%-partial hepatectomy. At 96 hr, the activity of TS was still significantly higher than the control, but the level of TK recovered to the control as shown in Table 1.

After pretreatment with phenobarbital (PB, 80 mg/kg body wt) for 3 days, rats were treated with CCl_4 and similar measurements were made after every 24 hr for the (PB + CCl_4) group. Results are shown in Table 1. The time course of plasma GOT and GPT elevation of the (PB + CCL_4) group was analogous to that seen in rats of the CCl_4 group (without pretreatment with PB) but the plasma GPT level at 48 hr was significantly higher in the (PB + CCl_4) group than in the CCl_4 group. Maximal activities of plasma GOT and GPT were observed at 48 hr and were as much as 214 and 130 times the control values respectively. Activity of TS in the liver of the (PB + CCl_4) group rats increased and was maximal at 72 hr in accord with the CCl_4 group. The maximal activity was nearly 20 times as much as the control value and twice that of the CCl_4 group. The time course of the activity of TK resembled that of TS. The maximal activity attained at 72 hr was 7 times the control and 1.7 times the CCl_4 group value. These differences compared with the control and the CCl_4 groups were statistically significant. These observations suggest that the liver damage is more extensive in the (PB + CCl_4) group than in the CCl_4 group. It is well documented that cytochrome P-450 is involved in the initial event of CCl_4 poisoning [1, 2], and administration of PB, which induces drug-metabolizing enzymes, enhances strikingly the hepatotoxic effect CCl_4 [26]. Therefore, our results are consistent with the idea that rats become hypersensitive to CCl_4 by pretreatment with PB. Only the pretreatment with PB and oil caused a significant elevation in the activity of TS, as shown in Table 1. Concerning the toxic effect of PB, Sorrell *et al.* [27] reported the accumulation of fat in the rat liver. However, liver cell destruction by PB seems insignificant on the basis of low levels of plasma GOT and GPT. The mechanism of the rise of TS activity by PB remains unexplained.

Recovery of the rat liver from damage caused by a single injection of CCl_4 is usually complete by 1 week [1, 28]. This is consistent with a prompt decrease in levels of plasma GOT and GPT as well as hepatic TS and TK from the maximal values. Because of the rapid injury followed by fast and complete recovery, CCl_4 poisoning may provide an excellent model system for the investigation of liver regeneration.

In summary, after 48 hr of administration of carbon tetrachloride (0.5 ml/100 g body wt), activities of liver thymidylate synthetase and thymidine kinase increased considerably and at 72 hr reached maximal levels, which were 10.5 and 5.4 times as much as the respective controls. These activities were comparable to the maximal levels attained by 67%-partial hepatectomy. Since these two enzymes are rate determining in DNA synthesis, the present results constitute biochemical evidence for liver regeneration. Pretreatment of the rats with phenobarbital appeared to result in more extensive damage after carbon tetrachloride poisoning when compared with the damage seen in rats without the pretreatment on the basis of enhanced levels of the thymidylate synthetic enzymes as well as plasma GPT.

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Trihexyphenidyl—Further evidence for muscarinic receptor subclassification

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Lately, an increased interest has been focused on the study of subtypes of muscarinic receptors. Recent reports [1–6] have shown that pirenzepine, a new synthetic muscarinic antagonist, binds with high affinity to some tissues and low affinity to others. Cerebral cortex, sympathetic ganglia, salivary glands and epithelial cells of the intestine* show high affinity pirenzipine binding and are thought to contain muscarinic M-1 receptors. Cerebellum, heart and muscle layers of the gastrointestinal tract* show low affinity pirenzepine binding and are thought to contain muscarinic M-2 receptors [1, 2]. New synthetic anticholinergic analogues have been identified which also have a tissue selectivity like that of pirenzepine and differentiate between M-1 and M-2 subtypes of muscarinic receptors [7]. Since a large number of compounds with antimuscarinic properties are available, it seems probable that some of those might show selectivity similar to that of pirenzepine. Many compounds with muscarinic receptor antagonist properties have been compared for binding affinity to muscarinic receptors of rat brain and guinea pig ileum [8]. Most antagonists show equal affinity for the two tissues, but trihexyphenidyl (Artane) displays a 10-fold higher affinity for the muscarinic receptors of the brain than for those of the ileum [8].

The aim of this investigation was to verify whether trihexyphenidyl shows selectivity for muscarinic receptor subtypes of the ileum and brain and to determine whether this compound, like pirenzepine, indicates that receptors of intestinal smooth muscle are different from those of intestinal epithelial cells.

Materials and methods

Chemicals. [^{3}H]Quinuclidinyl benzilate (QNB, 33.2 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA. (-)-Scopolamine methyl bromide, trihexyphenidyl hydrochloride and trizma hydrochloride and base were obtained from the Sigma Chemical Co., St. Louis, MO.

Methods. Trihexyphenidyl and methyl scopolamine displacement of [^{3}H]QNB binding were carried out on cerebral cortex, heart and colonic tissues obtained from male Sprague–Dawley rats (200–300 g). Colonic epithelial cells were vibrated off from the colon, and the remaining colonic tissue (which contains nerve plexuses in addition to the muscle fibers) was used as colonic smooth muscle. All the tissues were homogenized in Tris buffer (pH 7.4) and were centrifuged. Pellets were collected and used for binding assays. Binding experiments were conducted according to the procedure of Yamamura and Snyder [9]. The binding incubation mixture contained 1 nM [^{3}H]QNB, sufficient membrane protein to give approximately 1000 cpm bound in the absence of unlabeled competitors, and various concentrations of either methyl scopolamine or trihexyphenidyl in a final volume of 1.5 ml. Incubation was at 37° for 30–40 min. Bound and free [^{3}H]QNB were separated by filtration. The results from each experiment were plotted, and the IC_{50} values (concentration of unlabeled competitor that reduced [^{3}H]QNB binding to half of that observed with no competitor) were determined directly from the graphs. Means were calculated using log IC_{50} from three separate experiments. Statistical significance was evaluated using Duncan's multiple comparison test.

Results and discussion

Methyl scopolamine and trihexyphenidyl were studied for the ability to displace [^{3}H]QNB binding. A representative curve for each antagonist in the four tissues is shown in Fig. 1. The methyl scopolamine displacement curves were very close to each other in the four tissues. In contrast, the trihexyphenidyl displacement curves fell into two groups such that displacement occurred at a lower concentration of trihexyphenidyl in cerebral cortex and colonic epithelial cells than in heart and colonic smooth muscle. Finally, in all tissues, methyl scopolamine was somewhat more potent than trihexyphenidyl at displacing [^{3}H]QNB binding.

Means from replicate data are summarized in Table 1. For methyl scopolamine displacement of [^{3}H]QNB binding,

* X. Y. Tien, R. Wahawisan, L. J. Wallace and T. S. Gaginella, manuscript submitted for publication.