# Production of Interleukin-6 and Its Implication in Rats after Subcutaneous Injection of Carbon Tetrachloride

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Previously, we have reported that interleukin-6 (IL-6) administration reduces hepatic injury caused by carbon tetrachloride ($CCl_4$) and induces the production of antioxidant proteins, manganese superoxide dismutase and metallothionein. In the present study, we examined whether IL-6 was induced endogenously by $CCl_4$ administration in rats, and examined the relationship between the levels of IL-6 production and hepatic injury. Plasma samples were periodically collected after s.c. administration of 5 ml/kg of $CCl_4$ (50%, v/v, in corn oil). IL-6 was significantly produced at 1.5 hr after administration, peaked at 8 hr and gradually decreased thereafter. The activities of hepatic marker enzymes, alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) in plasma, gradually increased and peaked at 48 hr. As the ratio of the amount of corn oil to that of carbon tetrachloride was increased in the range of 1 : 0 to 1 : 8 (v/v), IL-6 induction was decreased, while ALT and SDH activities were augmented. When rats were treated with a pharmacological dose of dexamethasone (1 mg/kg), IL-6 production was decreased, but ALT and SDH activities were augmented. IL-6 expression immediately after $CCl_4$ administration is suggested to play some significant role in reducing hepatic injury. These findings should be thoroughly considered when the hepatic injury model is developed based on the s.c. administration of $CCl_4$.

**Key words** —— interleukin-6 production, carbon tetrachloride, hepatic injury, rat, amount of vehicle

## INTRODUCTION

Carbon tetrachloride ($CCl_4$) is known as a model compound for the study of hepatotoxicity. The compound is metabolized in the liver by cytochrome P450 (CYP2E1)[1] to the highly reactive trichloromethyl radical.[2] The metabolite causes lipid peroxidation in the endoplasmic reticulum, damage to the plasma membrane, and an alteration of calcium homeostasis prior to cell death.[3]

Interleukin-6 (IL-6) is a major regulator of the acute-phase response during inflammation.[4] Previously, we have reported that pretreatment with recombinant IL-6 or tumor necrosis factor (TNF)-$\alpha$ prevented the tissue damage and lipid peroxidation in the liver of carbon tetrachloride-treated rats, and that IL-6 induced the production of antioxidant proteins, manganese superoxide dismutase (Mn-SOD) and metallothionein (MT).[5,6] The protective activity of IL-6 against $CCl_4$-induced hepatotoxicity was supported by the findings that IL-6-deficient mice expressed liver injury and lipid peroxidation more intensely than normal mice upon $CCl_4$ administration.[7,8] These findings led us to examine whether IL-6 was endogenously induced in the $CCl_4$ hepatotoxicity model in relation to the potential reduction of hepatic injury.

## MATERIALS AND METHODS

**Reagents** —— Infinitely pure grade of $CCl_4$ (99.9%), corn oil, GPT-UV Test Wako, D-fructose, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) were purchased from Wako Biochemical Co. (Osaka, Japan). Dexamethasone (DEX) was from Nacalai Tesque Co. (Kyoto, Japan). Recombinant murine IL-6 was purchased from Seikagaku Corp. (Tokyo, Japan). RPMI-1640

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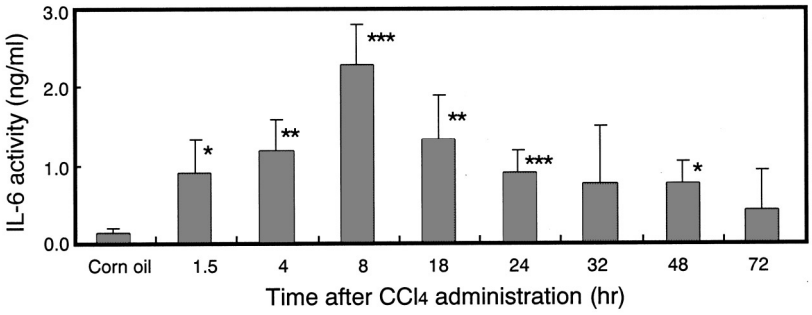


**Fig. 1.** Time Course of IL-6 Activity in Plasma of Rats Treated with $CCl_4$

Rats were treated s.c. with 5 ml/kg of $CCl_4$ (50%, v/v, solution in corn oil). Control group received an equal volume of corn oil. IL-6 activity of the experiment groups was measured at the times indicated and that of control group was measured at 8 hr. Data are expressed as mean ± S.D. ($n$=3–5). Significantly different from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

medium was purchased from Nissui Seiyaku (Tokyo, Japan) and fetal calf serum was obtained from JRH Biosciences A CSL Co. (Lenexa, KS, U.S.A.).

**Animals and Treatments** —— Male Wistar rats (5 weeks old) were obtained from SLC Japan (Shizuoka, Japan). The animals were fed a normal chow CE-2 (Japan CLEA, Tokyo, Japan) *ad libitum*, and housed for at least one week in an environmentally controlled room (light on 07:00–19:00) before use. Five ml/kg of $CCl_4$ (50%, v/v, solution in corn oil) was injected into the dorsal subcutaneous site of rats. DEX was suspended in sterilized 5% gum Arabic-saline and administered i.p. Blood samples were collected from the carotid artery into heparinized tubes under ether anesthesia and plasma was separated by centrifugation.

**Assay for IL-6 Activity** —— IL-6 activity in plasma was measured using an IL-6-dependent MH60.BSF2 cell line. Briefly, $10^4$ MH60.BSF2 cells were cultured with a serial dilution of samples in RPMI-1640 medium containing 10% heat-inactivated FCS in a flat-bottomed 96 well microtest plate in a humidified $CO_2$ incubator for 48 hr. Then, MTT solution was added and culture was carried out for another 2 hr.[9] After dissolving the product formazan into lysing buffer, absorbance at 590 nm was measured and transformed into amount of IL-6 by comparison to standard recombinant mouse IL-6.

**Assay for Enzyme Activity** —— To diagnose liver damages, the extent of leakage of alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) into plasma was estimated. ALT was quantified using the commercial kit, GPT-UV Test Wako, and SDH was determined as described by Gerlach and Hiby.[10]

**Statistical Analysis** —— Data were expressed as mean ± S.D. Significant differences between groups were analyzed using the Student's $t$-test. Statistical significance was accepted at the 0.05 level.

## RESULTS

### Time Course of IL-6 and Hepatic Enzyme Activities in Plasma from S.C. $CCl_4$-Treated Rats

Five ml/kg of $CCl_4$ (50%, v/v, solution in corn oil) was injected into the dorsal subcutaneous site of rats. IL-6 activity significantly elevated in plasma at 1.5 hr after injection and reached a peak at 8 hr (Fig. 1). Then, it decreased gradually but the low level of IL-6 activity remained significant even at 48 hr. Both ALT and SDH activities gradually increased to reach a peak at 48 hr (Fig. 2).

### Effect of the Ratio of the Amount of Vehicle to That of $CCl_4$ on IL-6 Induction and Hepatic Enzyme Release in Plasma

As some irritation at the $CCl_4$-injected site was thought to trigger IL-6 production, we examined the effect of the amount of vehicle for dissolving $CCl_4$ (5 ml/kg) on both IL-6 induction and hepatic injury. The greatest amount of IL-6 was induced when $CCl_4$ was administered without corn oil, and the amount of IL-6 produced became less as the ratio of the amount of corn oil to that of $CCl_4$ increased from 1 : 0 to 1 : 8 (Fig. 3). On the other hand, levels of ALT and SDH in plasma were the lowest upon $CCl_4$ administration without vehicle, and increased as the ratio of the amount of vehicle to that of $CCl_4$ was increased from 1:0 to 1:8 (Fig. 3). This indicated a reverse correlation between IL-6 production and leakage of ALT and SDH in s.c. $CCl_4$-treated rats.

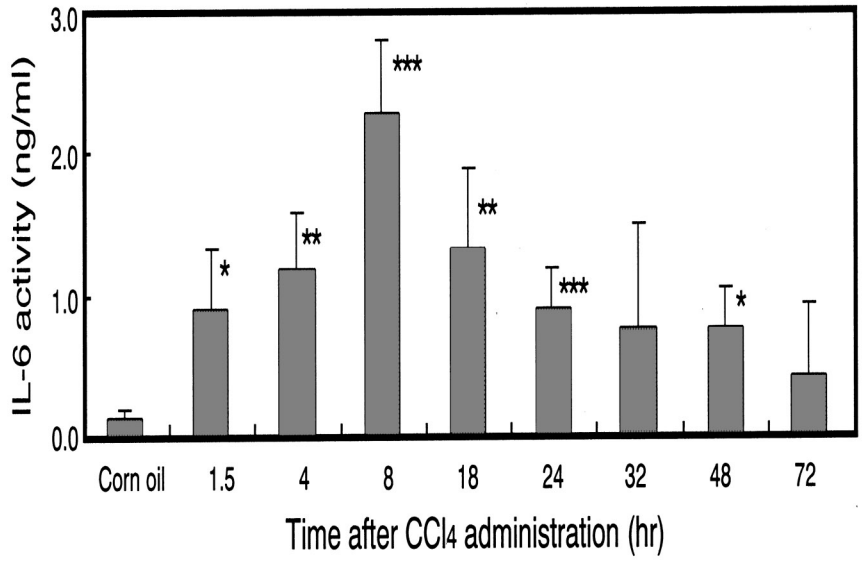


**Fig. 2.** Time Course of SDH and ALT Activities in Plasma of Rats Treated with $CCl_4$

Rats were treated s.c. with 5 ml/kg of $CCl_4$ (50%, v/v, solution in corn oil). Control group received an equal volume of corn oil. SDH and ALT activities of the experiment groups were measured at the times indicated and these of control group were measured at 8 hr. Data are expressed as mean ± S.D. ($n$=3–5). Significantly different from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

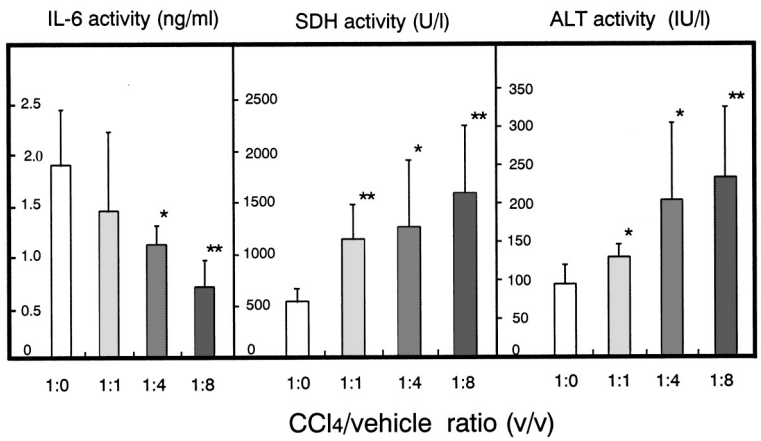


**Fig. 3.** Effect of the Ratio of the Vehicle to That of $CCl_4$ on IL-6 and SDH/ALT Activities

Rats were treated s.c. with $CCl_4$ (5.0 ml/kg) diluted with corn oil at various ratios in the range of 1 : 0–1 : 8. IL-6 activity was assayed at 8 hr, and SDH and ALT activities were assayed at 48 hr after $CCl_4$ administration. Data are expressed as mean ± S.D. ($n$=4–6). Significantly different from control, $*p < 0.05$, $**p < 0.01$.

### Effect of DEX on IL-6 Production and Hepatic Enzyme Release Induced by $CCl_4$ in Plasma

We examined whether the $CCl_4$-induced IL-6 production was suppressed by DEX pretreatment. The suppression of IL-6 production was virtually complete by pretreament with 1 mg/kg DEX (Fig. 4). On the other hand, amounts of ALT and SDH released by $CCl_4$ administration were enhanced several fold by DEX pretreatment at 1 mg/kg (Fig. 5). The results also indicate an inverse relationship between IL-6 production and leakage of ALT and SDH in the rats.

## DISCUSSION

In the present study, we showed that s.c. $CCl_4$ administration to rats induced IL-6 production and the level of IL-6 produced seemed to be inversely correlated with the hepatic enzyme level in plasma.

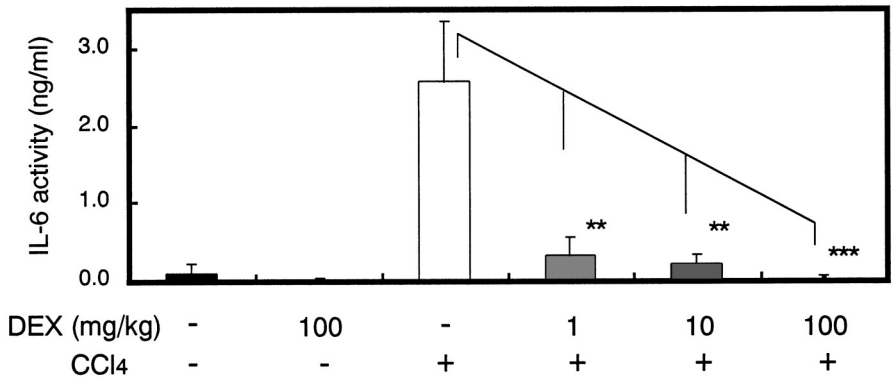


**Fig. 4.** Effect of DEX Pretreatment on $CCl_4$-Induced IL-6 Production

Rats were pretreated s.c. with DEX 6 hr before, and sacrificed 8 hr after s.c. administration of 5.0 ml/kg of $CCl_4$ (50%, v/v, solution in corn oil). Data are expressed as mean ± S.D. ($n$=3–4). Significantly different from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

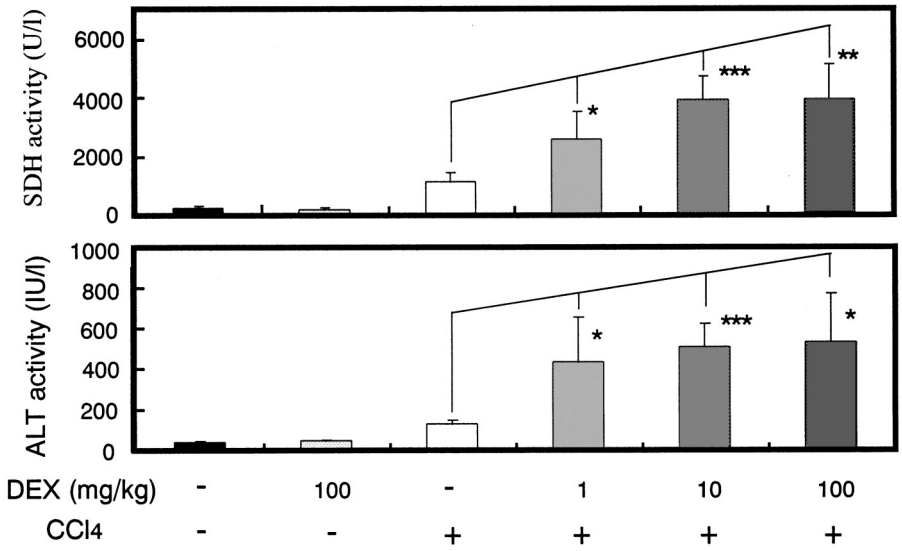


**Fig. 5.** Effect of DEX Pretreatment on $CCl_4$-Induced Hepatic Enzyme Release

Rats were pretreated s.c. with DEX 6 hr before, and sacrificed 24 hr after s.c. administration of 5.0 ml/kg of $CCl_4$ (50%, v/v, solution in corn oil). Data are expressed as mean ± S.D. ($n$=3–4). Significantly different from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

After s.c. administration of $CCl_4$, IL-6 was significantly elevated in plasma at 1.5 hr and peaked at 8 hr (Fig. 1), preceding liver injury as shown by the release of hepatic marker enzymes ALT and SDH, which reached a peak at 48 hr (Fig. 2). As the amount of vehicle was increased, IL-6 induction by $CCl_4$ was reduced and plasma ALT and SDH levels were enhanced (Fig. 3). Although the mechanism by which IL-6 was induced by $CCl_4$ remained unclear, it was speculated that $CCl_4$ might give some stimuli to the site of injection, and the stimuli might increased in intensity when $CCl_4$ was administered with a smaller amount of vehicle. As IL-6 induction was thought to be mediated by adrenaline released by $CCl_4$ simulation, we investigated the effect of $\beta$-adrenergic receptor blocker *dl*-propranolol (15 mg/kg, i.p.) on the $CCl_4$-induced IL-6 induction. Although adrenaline-induced IL-6 production was completely suppressed by *dl*-propranolol,[11] $CCl_4$-induced IL-6 production was not affected by the treatment (data not shown).

Glucocorticoids are potent inhibitors of IL-6 production induced by various stimuli such as lipopolysaccharide or TNF.[12,13] $CCl_4$-treated IL-6 production was also inhibited by DEX pretreatment (Fig. 4). Plasma ALT and SDH levels were, however, enhanced markedly by DEX pretreatment in the $CCl_4$-treated rat (Fig. 5). It seems unusual that a pharmacological level of DEX augments the inflam-

mation of tissues. This phenomenon might mean that the amounts of some soluble factors such as IL-6 or inflammatory cells, which have protective function against tissue injury, were decreased by DEX.

Our and other groups have reported that IL-6 treatment suppresses $CCl_4$-induced liver injury, which developed through radical production, in rats and mice.[6,7,8] MT is known to be a protective against the toxicity of heavy metals and to regulate the metabolism of the essential metals such as zinc or copper.[14] Moreover, this sulfur-rich protein has been suggested to be involved in scavenging radicals.[15] It was reported that an injection of $CCl_4$ induced MT synthesis,[16] and that an MT inducer inhibited $CCl_4$ induced hepatotoxicity.[17] SODs also function to scavenge and dismutate the superoxide free radicals.[18] IL-6 induces Mn-SOD[6,19] and MT in the liver.[5,6] Therefore, these antioxidant proteins are suggested to play an important role in suppressing the development of hepatic injury by $CCl_4$. DEX and IL-6 synergistically increase the synthesis of Mn-SOD[19] and MT[20] in hepatocyte cultures. These effects are thought to be mediated by the up-regulation of a 80 kDa IL-6 receptor by DEX[21] and/or by the up-regulation of a 130 kDa glycoprotein involved in signal transduction (gp130).[22] However, since DEX was administered prior to $CCl_4$ in the present study, such synergic effect might not be expressed. In addition, TNF-$\alpha$, which is able to induce IL-6 production,[23] was found to induce the production of Mn-SOD and MT in rats,[6] However, we could not detect TNF activity in plasma, at least in the early hours (1.5–8.0 hr) after 50% $CCl_4$ (5 ml/kg, v/v) in corn oil was injected s.c. (data not shown), which was different from the report that a substantial level of TNF-$\alpha$ was induced.[24] The reasons why we could not detect TNF activity might be the difference in sensitivity of the assay method or the suppression of TNF-$\alpha$ gene expression by IL-6 produced.[25]

We demonstrated in the present study that IL-6 was expressed early when $CCl_4$ was s.c. injected without or with a small amount of vehicle, and that the level of IL-6 induction had an inverse relationship with hepatic injury. The endogenously induced IL-6 was suggested to suppress the development of hepatic injury. These findings presented here should be noted in using the $CCl_4$-induced hepatotoxicity model.

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