Biochemical Pharmacology, Vol. 21, pp. 1697–1702. Pergamon Press, 1972. Printed in Great Britain.

CARBON TETRACHLORIDE-INDUCED PROTECTION AGAINST CARBON TETRACHLORIDE TOXICITY

THE ROLE OF THE LIVER MICROSOMAL DRUG-METABOLIZING SYSTEM*

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(*Received* 28 *October* 1971; *accepted* 7 *January* 1972)

Abstract—Intragastric administration to rats of a small non-lethal dose of carbon tetrachloride produces a dramatic protective action against subsequently administered large and ordinarily lethal doses of the same liver poison. Resistance to the lethal effect of carbon tetrachloride sets in by 12 hr and is fully developed by 24 hr. Protection lasts for 3–5 days after which it gradually subsides. Activity of liver microsomal aminopyrine demethylase and cytochrome P-450 concentration were followed throughout the course of development of the protection phenomenon. Activity of aminopyrine demethylase and cytochrome P-450 concentration began to fall immediately after administration of the small initial dose of carbon tetrachloride. At 24 hr, when protection was complete, the activity of this enzyme system was about one-fourth of control levels. The depression lasted for 4 days, which was followed by gradual recovery. The exact parallelism between depression of liver microsomal mixed function oxidase activity and resistance to the lethal effects of carbon tetrachloride affords strong evidence for the view that metabolism of carbon tetrachloride by the liver microsomal drug-metabolizing system is a necessary prerequisite for the toxicity of this liver poison.

FLOERSHEIM[1] reported that, after administration of small doses of carbon tetrachloride (CCl_4) to mice, within 24 hr the animals were completely resistant to double an LD_{95} of phalloidin. Phalloidin is a toxic principle from the white mushroom, *Amanita phalloides*. More recently, Dambrauskas and Cornish[2] observed that, after administration of non-lethal doses of carbon tetrachloride, rats became highly resistant to the lethal effects of carbon tetrachloride itself. Development of resistance to carbon tetrachloride after carbon tetrachloride administration has been confirmed[3] and subjected to close study in this laboratory.[4]

Lipoperoxidative decomposition of the hepatic endoplasmic reticulum is an early event in carbon tetrachloride liver injury.[5] This process is thought to be the critical first step on the path leading to fatty liver, hepatocellular necrosis, and death of the animal.[6,7] The lipid peroxidation in turn is believed to be initiated by free radicals arising from metabolism of carbon tetrachloride somewhere along the mixed function oxidase chain of enzymes in the liver endoplasmic reticulum.[8,9] Numerous reports have indicated that carbon tetrachloride depresses activity of the hepatic microsomal drug-metabolizing system.[10–13] It follows that if hepatic microsomal drug-metabolizing activity is intimately involved in the toxigenesis of carbon tetrachloride liver injury,

* This investigation was supported in part by Grant No. 5-RO1-AMO1489 from the National Institute of Arthritis and Metabolic Diseases, N.I.H., U.S.P.H.S.

then carbon tetrachloride-induced depression of this system should parallel, in time, the development of the carbon tetrachloride self-protection phenomenon. It is the purpose of this communication to show that development of resistance to carbon tetrachloride is, indeed, directly related to depression of NADPH-linked microsomal drug metabolism.

METHODS

Procedures on animals. Male, Sprague–Dawley strain of rats, 200–300 g body weight were used. The animals were housed in wire-bottom cages and allowed free access to water and standard laboratory chow (Wayne Lab Blox) except for various periods preceding and following carbon tetrachloride administration (see below). Carbon tetrachloride (Mallinckrodt, AR) dissolved in mineral oil (Fisher, USP) was given by stomach tube under light ether anesthesia. Control animals received an equal volume of the vehicle (0·5 ml mineral oil/100 g body weight). The protection phenomenon was elicited according to a procedure worked out in this laboratory.* Animals were fasted overnight prior to administration of the small, protective dose of carbon tetrachloride, which was 25 μl CCl_4/100 g body weight. The challenging dose was 400 or 500 μl CCl_4/100 g body weight. Animals were maintained in the fasting condition when the time interval between administration of the protective and challenging doses was 12 hr or less. For longer time intervals, food was offered 6 hr after administration of the protective dose, but was again withdrawn for at least 6 hr before administration of the challenging dose. In all cases, food was offered *ad lib.* 6 hr after administration of the challenging dose. Water was available *ad lib.* throughout the experiment.

Enzyme assays. Rats were killed by cervical section and bled. The liver was homogenized immediately with a Potter–Elvehjem homogenizer in ice-cold saline buffered at pH 6·5 with 0·05 M phosphate (Na-salt). The homogenate was centrifuged at 2700 *g* for 10 min to sediment the nuclear and mitochondrial fractions. The supernatant fraction was centrifuged at 100,000 *g* for 30 min. The sedimented microsomal pellet was resuspended in saline–phosphate buffer medium by gentle homogenization. Aliquots of the final microsomal suspension were used immediately to measure NADPH-cytochrome *c* reductase, aminopyrine demethylase and cytochrome P-450.

Microsomal NADPH-cytochrome *c* reductase activity was measured at 25° by observing the increase in optical density at 550 nm produced by reduction of cytochrome *c*. The final reaction volume was 3·0 ml, and it contained 0·10 m-mole phosphate buffer, pH 7·4, 1·0 μmole KCN, 0·08 m-mole nicotinamide, 0·3 μmole NADPH (omitted in the control reaction), 0·1 μmole oxidized cytochrome *c*, and microsomal material containing approximately 0·04 mg protein. Enzyme activity was linear with respect to microsome concentration, and reaction velocity was linear for several minutes. NADPH-cytochrome *c* reductase activity was calculated as the change in absorbancy at 550 nm/min/mg microsomal protein.

Drug-metabolizing activity of the microsome preparation was determined by measuring aminopyrine demethylase. Flasks containing 40 μmoles aminopyrine and microsomes containing 6 mg protein were incubated at 38° for 15 min. A continuous supply of NADPH was assured by addition of 1·5 μmoles NADP, 20 μmoles nicotinamide, 48 μmoles isocitrate, 40 μmoles $MgCl_2$, and 50 mg isocitric dehydrogenase

* G. Ugazio, R. R. Koch and R. O. Recknagel, unpublished observations.

(Sigma, type I). Total reaction volume was 8·0 ml. The reaction was stopped by adding 1·0 ml of 15% trichloroacetic acid to 2·0-ml aliquots of the mixture. After sedimentation of the precipitated protein by centrifugation, content of formaldehyde was determined according to the procedure of Nash.[14] Production of formaldehyde was proportional to the concentration of microsomes, and it was linear during the 15-min incubation period. Enzyme activity was calculated as millimicromoles of formaldehyde produced in 15 min per milligram of microsomal protein, and corrected for formaldehyde present before start of the incubation.

Microsomal cytochrome P-450 concentration was determined according to Omura and Sato.[15] Protein content of the microsomes was determined by the method of Lowry *et al.*[16]

RESULTS

For the first 6 hr after administration of a small dose of carbon tetrachloride, rats succumb to the lethal effects of a large, challenging dose of the same liver poison (Fig. 1).* However, by 24 hr the rats are completely resistant to an LD_{95} of CCl_4. The protection lasts for 3 days, after which it gradually subsides.

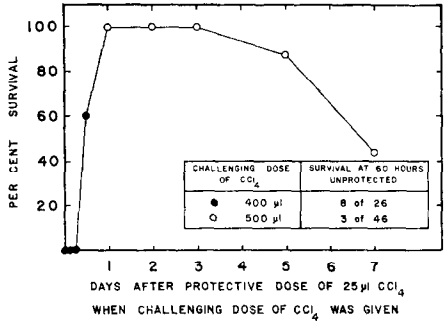

FIG. 1. Development of resistance to carbon tetrachloride toxicity after administration of a small dose of carbon tetrachloride (adapted after Ugazio *et al.*, unpublished observations).

Immediately after administration of a small protective dose of carbon tetrachloride, activity of liver microsomal aminopyrine demethylase begins to fall (Fig. 2). By 24 hr, activity of this enzyme system is one-fourth of control levels. It remains depressed for 4 days, after which it gradually recovers. The detectable level of cytochrome P-450 faithfully parallels activity of aminopyrine demethylase during the entire 7-day period of the development of resistance and recovery of susceptibility to carbon tetrachloride. In contrast, activity of NADPH-cytochrome *c* reductase is virtually unaffected during the same period of 7 days following administration of the protective dose of carbon tetrachloride.

DISCUSSION

The protection phenomenon reported here confirms and extends the report of Dambrauskas and Cornish.[2] These workers reported on tolerance development after exposure of rats to carbon tetrachloride vapors—and in one experiment after intra-

* Adopted from data of G. Ugazio, R. R. Koch and R. O. Recknagel, unpublished observations.

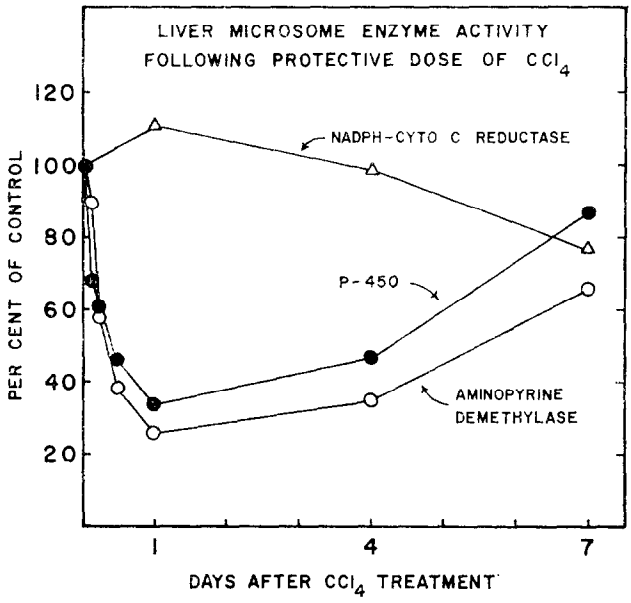

FIG. 2. Liver microsome enzyme activity following a protective dose of carbon tetrachloride (25 μl/100 g body weight, intragastric). Data for each time point came from five experimental and five control rats. Control averages: TPNH-cytochrome *c* reductase, 0·438 ΔE_{550}/min/mg microsomal protein; aminopyrine demethylase, 29 nmoles HCHO produced/15 min/mg microsomal protein; cytochrome P-450, 0·93 nmol/mg microsomal protein.

gastric administration of a relatively large dose of carbon tetrachloride. When the protective dose is small and when it is given intragastrically, development of protection is more sharply defined than when the protective dose of carbon tetrachloride is via inhalation for 6 hr.[2]

Ugazio *et al.*[3] suggested that development of resistance to large and ordinarily lethal doses of carbon tetrachloride was due to the capacity of the initial small dose to depress liver microsomal drug-metabolizing activity. Their suggestion was based on the observation that 48 hr after administration of the protective dose, when rats are completely resistant to an LD_{95} of CCl_4, hexobarbital sleeping time was increased more than 3-fold, and conversion of intragastrically administered $^{14}CCl_4$ to expired $^{14}CO_2$ was depressed to one-half the rate for control rats.

Long-term depression of microsomal drug-metabolizing activity after administration of carbon tetrachloride was noted by Barker *et al.*[11] and by Dingell and Heimberg.[10] However, the dose of carbon tetrachloride was 10-fold greater than the small dose which confers the remarkable protection depicted in Fig. 1. Furthermore, if the suggestion of Ugazio *et al.*[3] is right, it follows that development of resistance and depression of liver microsomal drug-metabolizing activity should show a close parallelism in time. The data of Figs. 1 and 2 show the required parallelism. During the first 24 hr when resistance to carbon tetrachloride is developing, there is a steady decline in activity of aminopyrine demethylase and microsomal content of cytochrome P-450. This enzyme system remains depressed during 4 days coincident with complete resistance of the rat to carbon tetrachloride. As microsomal mixed function oxidase

activity gradually recovers, the rat once again becomes susceptible to whole-body carbon tetrachloride poisoning.

Activity of the flavoprotein component of the NADPH-linked microsomal drug-metabolizing system, measured as NADPH-cytochrome *c* reductase activity, was not significantly altered for 4 days after administration of the protective dose of carbon tetrachloride (Fig. 2). The fact that activity of this flavoprotein is not affected during the period when complete tolerance to the lethality of carbon tetrachloride is observed supports the view[9] that this flavoprotein is not directly involved in carbon–chlorine bond cleavage of carbon tetrachloride. On the other hand, marked depression of aminopyrine demethylase and cytochrome P-450 during the period of resistance to carbon tetrachloride, coupled with the observation[3] that conversion of $^{14}CCl_4$ to $^{14}CO_2$ is depressed at the same time, clearly indicates that the site along the microsomal electron transport chain where carbon–chlorine bond cleavage takes place is remote from the locus of the flavoprotein. This conclusion is contrary to a view offered by Slater and Sawyer.[17]

In a series of papers[18–20] data were presented which suggested the possibility that some of the toxic effects of carbon tetrachloride may not be related to carbon tetrachloride metabolism or attendant lipid peroxidation. A critical analysis of this work has been made.[21] From the data presented in Fig. 1, it is clear that a normally lethal dose of carbon tetrachloride has been rendered non-lethal by the simple expedient of administration of one-twentieth of an LD_{95} 24 hr earlier. If a solvent action or some other action not involving carbon tetrachloride metabolism were a significant feature of carbon tetrachloride lethality, then an LD_{95} ought to kill at least some of the rats. In actual fact, the rats are completely resistant. This argues strongly against any significant role for a non-chemical action of carbon tetrachloride in the mechanism of carbon tetrachloride lethality.

The work reported here is significant from another point of view. The protective dose of carbon tetrachloride appears to have a remarkably localized and surprisingly large depressant effect on discrete components of the liver microsomal NADPH-linked electron transport chain. This section of the drug-metabolizing system appears to be the site for carbon–chlorine bond cleavage and resultant lipid peroxidation.[8] The lipid peroxidation in turn appears to be the key chemical event on the path leading to cellular necrosis and eventual death of the animal.[6,7] It is obvious from the study here presented that this critical site in the liver endoplasmic reticulum can be subjected to selective injury. The protection phenomenon as reported here may thus provide a useful model system for further investigative work on fundamental questions of cellular injury and repair.[4]

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