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Resiliency to Amplification of Carbon Tetrachloride Hepatotoxicity by Chlordecone during Postnatal Development in Rats

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ABSTRACT. The interactive hepatotoxicity of CCl_4 and chlordecone, at an individually nontoxic dosage, was studied in neonatal and young developing rats. The well-documented amplification of CCl_4 (100 μL/kg) hepatotoxicity and lethality by prior dietary exposure to chlordecone (10 ppm, for 15 d) was absent in neonatal and developing rats through 35 d of age. The chlordecone-potentiated hepatotoxicity and lethality of CCl_4 was partially expressed in 45-d-old rats and fully expressed in 60-d-old rats. Although hepatic microsomal cytochrome P-450 content in 2- or 5-d-old rats was significantly lower than that in older age groups, the cytochrome P-450 content was not significantly different between 35-, 45-, and 60-d-old chlordecone-treated rats. During postnatal development, the ongoing hepatocellular proliferation declined in a biphasic manner, more rapidly up to 20 d and slowly thereafter, as indicated by ^{3}H-thymidine incorporation in hepatic nuclear DNA. This pattern of postnatal liver proliferation and growth was not altered by exposure to chlordecone. *In vivo* metabolism of CCl_4, in terms of $^{14}CO_2$ production derived from $^{14}CCl_4$ and $^{14}CCl_4$ metabolites bound to hepatic tissue, was not significantly different between 35-, 45-, and 60-d-old chlordecone-treated rats, whereas CCl_4-stimulated hepatocellular regeneration in 35-d-old chlordecone-treated rats was significantly higher than in 45- or 60-d-old chlordecone-treated rats, as indicated by ^{3}H-thymidine incorporation into hepatic DNA and histomorphometric analysis. These data suggest that the absence of potentiation of CCl_4 toxicity by chlordecone in postnatally developing rats is well correlated with the presence of ongoing and stimulatable hepatocellular regenerative activity. (*Pediatr Res* 33: 225-232, 1993)

Abbreviations

CD, chlordecone
PB, phenobarbital
^{3}H-T, ^{3}H-thymidine
ALT, alanine transaminase
ND, normal diet
TGF, transforming growth factor

The remarkable potentiation of CCl_4 hepatotoxicity by prior exposure to nontoxic levels of CD (10 ppm in diet for 15 d) is well documented in male and female adult rats, as indicated by greatly increased hepatotoxicity and lethality of CCl_4 at ordinarily nontoxic doses in CD-treated rats (1-3). A number of candidate mechanisms for this interaction have been considered (4). Increased bioactivation of CCl_4 followed by increased lipid peroxidation is the foremost of these mechanisms in view of their wide acceptance with respect to PB, alcohol, ketone, and other xenobiotic-induced enhancement of CCl_4 toxicity (5-7). Studies of *in vitro* (8) and *in vivo* (9) metabolism of CCl_4 in rats indicated that, although enhanced metabolism of CCl_4 induced by cytochrome P-450 inducers such as PB and CD resulted in increased hepatotoxic effects of CCl_4 initially, the alterations in CCl_4 metabolism in PB- and CD-pretreated rats were not paralleled by the increase of CCl_4 lethal effects. These studies revealed that a 3-fold increase in CCl_4 metabolism with a statistically nonsignificant 1.7-fold increase in CCl_4 lethality for PB rats was contrasted with a 2-fold increase in CCl_4 metabolism with 67-fold increase in CCl_4 lethality for CD-treated rats. Therefore, enhanced metabolism of CCl_4 did not adequately explain the mechanism of CD-potentiated CCl_4 hepatotoxicity. Other possible mechanisms including increased lipid peroxidation and covalent binding of CCl_4 metabolites and the estrogenic property of CD have also been demonstrated not to be adequate explanations for the specificity and magnitude of CD-induced potentiation of CCl_4 hepatotoxicity and lethality (10, 11).

On the other hand, evidence from histomorphometric studies (12, 13) and from subsequent investigations using ^{3}H-T pulse-labeling and autoradiography techniques and the partially hepatectomized animal model (14-17) are supportive of a novel hypothesis (4) that normally hepatocellular regeneration and repair of the hepatolobular architecture enable the animals to overcome and recover from injury initiated by a low dose of CCl_4. These studies (14-17) also revealed that suppression of hepatocellular regeneration and tissue repair are responsible for the amplified hepatotoxicity of the same low dose of CCl_4 by prior exposure to CD. The suppressed CCl_4-stimulated hepatocellular regeneration and tissue repair in CD-treated rats has been shown to be due to a precipitous depletion in hepatocellular ATP (18). Significant attenuation of CD-amplified CCl_4 hepatotoxicity afforded by administration of (+)-cyanidanol, which significantly restored cellular regeneration and tissue repair as a consequence of increased hepatic ATP levels (19, 20), provided further supportive evidence for the hypothesis.

Although most hepatocytes in adult rats are in a nonproliferating quiescent state, during postnatal development hepatocytes actively proliferate and liver growth occurs. In the present study, we used newborn and young animals as an additional model to test the role of active cell division in resistance to potentiated hepatotoxicity of CCl_4 by CD.

During postnatal development, the newborn and young animals are usually more sensitive than adults to many drugs and toxicants (21-26). However, the newborns are less sensitive to chemicals like CCl_4 whose toxicity is dependent on prior metabolic activation to the toxic species (7, 27, 28), because they have

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a less than fully developed cytochrome P-450 system. To consider the differences in CCl_4 bioactivation, which might result in the differences in resiliency to the potentiation of CCl_4 hepatotoxicity by CD, *in vivo* metabolism of CCl_4 was also investigated in rats at various ages with or without the CD pretreatment.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all chemicals and enzyme kits used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-T (sp act 20 Ci/mmol) and $^{14}CCl_4$ (99% purity, 2.8 mCi/mmol) were obtained from New England Nuclear (Boston, MA). $^{14}CCl_4$ was prepared with unlabeled CCl_4 in corn oil to achieve a concentration of CCl_4 (100 μL/mL) with a sp act of 0.013 mCi/mmol. Analytical grade CCl_4 and scintillation fluid, Scintiverse E SX 16–4, were purchased from Fisher Scientific Company (Baton Rouge, LA). For lethality and hepatotoxicity studies, CCl_4 was dissolved in corn oil at concentrations of either 50 or 100 μL/mL for intraperitoneal administration.

Animals. Male Sprague-Dawley rats at the age of 20, 30, or 45 d and pregnant female rats at d 7 of gestation were purchased from Charles River Breeding Laboratories (Wilmington, MA) and maintained in our central animal facilities away from any contaminants. All experiments were performed humanely and with the approval of the Institutional Animal Care and Use Committee.

The male rats were housed four per cage and pregnant female rats two per cage over untreated corn cob bedding under a 12-h photoperiod, 50–80% humidity, at 21°C. Water and powdered Purina rodent chow (diet no. 5001, Purina Chow Co., St. Louis, MO) containing 10 ppm CD were provided *ad libitum* to male rats at the age of 20, 30, or 45 d for 15 d. This diet was prepared as previously described by Curtis and Mehendale (29). On d 16 of the dietary protocol (when the rats reach 35, 45, and 60 d of age, respectively), they were used for lethality, $^{14}CCl_4$ metabolism, ^{3}H-T incorporation, or other studies. Pregnant female rats were provided with a similarly prepared CD diet (30 ppm) for 15 d, starting from 7 or 10 d of gestation or 5 d postparturition such that their 2-, 5-, and 20-d-old offspring would have been also exposed to CD for 15 d, either transplacentally or through milk feeding. On d 18 of gestation, the pregnant rats were separated from each other and maintained in individual cages. On d 16 of the dietary protocol, the 2-, 5-, and 20-d-old rats were used for lethality, ^{3}H-T incorporation, and hepatic CD deposition measurements (in some studies, the 5-d-old group was omitted). Control rats were maintained on a similarly prepared powdered diet without the addition of CD. Only male rats were used for experimental measurement, except for the 2-d-old group, in which blood or hepatic tissue samples from two to three pups had to be pooled for a single measurement. Because no significant difference in the parameters measured was found between males and females in that group, data for males and females were pooled to reduce the number of pups needed.

Lethality. Lethality induced by a single dose of CCl_4 (100 μL/kg) was determined in 2-, 20-, 35-, 45-, and 60-d-old rats with or without CD pretreatment. After the rats were challenged with an intraperitoneal injection of CCl_4, they were observed for 14 d and lethality was recorded. Carbon tetrachloride was prepared in corn oil in a concentration of 50 μL/mL for 2- and 20-d-old rats and 100 μL/mL for rats in other age groups.

Microsomal cytochrome P-450. Hepatic microsomal protein and cytochrome P-450 content in 2-, 5-, 20-, 35-, 45-, and 60-d-old rats with or without CD pretreatment were determined using the methods of Lowry (30) and Omura and Sato (31), respectively.

Evaluation of CCl_4 hepatotoxicity. Rats were killed under diethyl ether anesthesia, and hepatotoxicity was assessed at a series of time points after the challenge with a single intraperitoneal dose of CCl_4 (100 μL/kg) by assessing elevation of serum ALT (EC 2.6.1.2.) following the method of Reitman and Frankel (32) and by histopathologic examination of the liver tissue for cellular necrosis. Volume density of hepatocytes with each of the various morphologic features was separately determined according to Weibel *et al.* (33). Control groups were injected with corn oil.

Estimation of CD deposition in hepatic tissue. To validate our CD pretreatment of the infants through dietary exposure to the dams, the CD concentrations in newborn and young rat hepatic tissue were measured by gas chromatography as described by Curtis and Mehendale (34).

^{3}H-T incorporation into liver nuclear DNA. Together with the evidence from mitotic activity, ^{3}H-T incorporation into liver nuclear DNA was measured as an index of hepatocellular regenerative activity, following the procedures described previously (35). ^{3}H-T was administered at a dose of 200 μCi/kg 2 h before the animals were killed. The procedure used for isolation of liver nuclear DNA was that described by Chang and Looney (36). The DNA content was measured with the diphenylamine reaction as described by Burton (37). ^{3}H-T incorporation data were expressed as cpm/mg DNA.

In vivo metabolism of $^{14}CCl_4$. The procedure for estimation of *in vivo* metabolism of $^{14}CCl_4$ in 35-, 45-, and 60-d-old rats with or without CD pretreatment was the same as that described earlier (9, 38). Briefly, after injection of $^{14}CCl_4$ at a dose of 100 μL/kg, the rat was put in a glass metabolic chamber. The expired air was successively drawn at an approximate flow rate of 800 mL/min through two traps containing 10 mL of toluene to collect unmetabolized $^{14}CCl_4$ and a third trap containing 10 mL of NaOH to collect $^{14}CO_2$ derived from $^{14}CCl_4$. Each of the traps was immersed in an ice water bath maintained at 4°C. Trap contents were removed for analysis at hourly intervals for 6 h. The radioactivity of collected $^{14}CCl_4$ and $^{14}CO_2$ was counted in a Packard 2200CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT) and expressed as dpm. Upon completion of the 6-h collection interval, the liver was surgically removed while the rat was under diethyl ether anesthesia for the estimation of $^{14}CCl_4$ metabolites bound to liver tissue.

Total hepatic ^{14}C and $^{14}CCl_4$ metabolites bound to hepatic tissue were determined as previously described (38) and expressed as dpm/g liver.

Statistics. Data of measured variables from various age groups at the corresponding time points were subjected to conventional one-way analysis of variance. Duncan's multiple comparison was used to determine the significance of differences between groups when one-way analysis of variance indicated a significant F value. The χ^2 test was used to determine the significance of the difference in mortality between groups. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

Lethality. The mortality induced by a single dose of CCl_4 (100 μL/kg) in 2-, 20-, 35-, 45-, and 60-d-old rats with or without CD pretreatment during 14 d is shown in Table 1. No mortality was observed in control rats at any age and in CD-treated rats at the age of 2, 20, and 35 d. Mortality first appeared in the 45-d-old group treated with CD and was further significantly increased in 60-d-old CD-treated rats, indicating that the toxicity of a nontoxic dose of CCl_4 was greatly amplified by CD only in certain age groups. Animal death occurred only during 36 to 72 h after the CCl_4 administration.

CD Deposition in Hepatic Tissue. Hepatic concentrations of CD in newborn and young rats exposed to a CD diet for 15 d are listed in Table 2. These values are comparable to the hepatic CD concentration in adult rats pretreated with 10 ppm dietary CD, 51.6 ± 0.9 μg/g liver, which resulted in remarkable potentiation of hepatotoxic and lethal effects of an ordinarily nontoxic dose of CCl_4 (34). These values also suggest that our dietary pretreatment of the newborn or young rats via dietary exposure of the mother rats is effective and that the lack of CD-potentiated

Table 1. *Lethality within 14 d after CCl_4 administration in rats at different ages**

Age (d)	ND ($n = 10$)†	CD diet (10 ppm)
2	0	0 (10)†
20	0	0 (10)
35	0	0 (32)
45	0	24%‡ (25)
60	0	90%§ (10)

* After the CD preexposure regimen, the rats were injected intraperitoneally with a single dose of 100 μL CCl_4/kg body weight in corn oil vehicle.

† The numbers in parentheses are total number of animals treated.

‡ $p < 0.05$ compared with 0.

§ $p < 0.01$ compared with 0.

lethal effects of CCl_4 in 2- and 20-d-old rats is unlikely to be due to insufficient deposition of CD in hepatic tissue.

Hepatic Microsomal Cytochrome P-450. The hepatic microsomal cytochrome P-450 content increased with age (Fig. 1). At the ages of 2 and 5 d, the cytochrome P-450 level was significantly lower than in the older groups. CD treatment seemed to accelerate the rate of increase in cytochrome P-450 with age. On d 20, cytochrome P-450 in CD-treated rats reached the plateau level and it was significantly higher than in the control rats. By 35 d after birth, the cytochrome P-450 level started plateauing in both control and CD-treated rats. CD pretreatment is known to increase cytochrome P-450 content, and this increase was evident at all ages.

Hepatotoxicity of CCl_4. Within 24 h after the injection of CCl_4, the ALT level in 2-d-old rats was fairly stable regardless of the pretreatment, indicating a lack of hepatotoxicity (Fig. 2). The ALT level in 20-d-old rats with or without CD pretreatment also remained stable after the administration of CCl_4 (data not shown). In contrast to this, the combination of CD + CCl_4 resulted in significantly elevated ALT levels in 35-, 45-, and 60-d-old rats as early as 2 or 6 h after the CCl_4 administration, whereas the same dose of CCl_4 had no effect on the ALT level in control diet–fed rats (Fig. 3*a* and *b*). The elevated ALT levels were not significantly different among 35-, 45-, and 60-d-old CD-treated rats at the 2 and 6 h time points, indicating that the initial liver injury in these groups was very similar. By 24 and 48 h after CCl_4 injection, the ALT levels in 45- and 60-d-old CD-treated rats were further dramatically elevated, whereas the ALT level in 35-d-old CD-treated rats did not increase much more (Fig. 3*c* and *d*), suggesting that the progression of the initiated liver injury by CCl_4 in 35-d-old CD-treated rats was different from that in 45- or 60-d-old CD-treated rats. It should be noted that animal death occurs in these two groups after 36 h, and the ALT values were measured only from the surviving rats. Therefore, the ALT level in 45- or 60-d-old CD-treated rats 48 h after the CCl_4 administration may even be underestimated here.

^{3}H-T Incorporation into Liver Nuclear DNA. In the absence of CCl_4 administration, the ^{3}H-T incorporation rate decreased significantly with the increase of age regardless of the dietary treatment (Fig. 4). The 2- and 5-d-old rats had much higher ^{3}H-T incorporation rates than other age groups and the incorporation rates at 20 or 35 d were still significantly higher than in the older groups (Fig. 4, *insert*). CD treatment had no significant effects on ^{3}H-T incorporation for any of these age groups.

Because 35-, 45-, and 60-d-old CD-treated rats had similar initial liver injury 6 h after the administration of CCl_4 but had a dramatically different final outcome of toxicity, we determined levels of ^{3}H-T incorporation into hepatic nuclear DNA in these groups at 2, 6, 24, and 48 h after CCl_4 as an index of tissue repair activity.

Two h after CCl_4 administration, ^{3}H-T incorporation was significantly increased in 45- and 60-d-old ND-fed rats, but not in CD-treated rats, as compared with the respective corn oil-injected control groups (Fig. 5*a*). The administration of CCl_4 also resulted in an elevation of ^{3}H-T incorporation in 35-d-old ND-fed rats, but the increase was not statistically significant. This is probably due to the higher rate of ^{3}H-T incorporation in 35-d-old control rats in comparison to the older groups. The ^{3}H-T incorporation level in 35-d-old ND-fed or CD-treated rats challenged with CCl_4 was significantly higher than in the older ones receiving the same treatment (Fig. 5*a*), and this trend was also evident at 6 h after CCl_4 administration (Fig. 5*b*). By 24 h after CCl_4 injection, a significant increase in ^{3}H-T was observed in 35- and 45-d-old rats but not in 60-d-old CD-treated rats as compared with the control or the corn oil–injected CD-treated rats (Fig. 5*c*). The increased ^{3}H-T incorporation rate in 35-d-old CD-treated rats was much higher than observed in older age groups challenged with the same dose of CCl_4. The administration of CCl_4 induced significant increases in ^{3}H-T incorporation by 48 h in both ND-fed and CD-treated rats at all ages studied (Fig. 5*d*). However, because ^{3}H-T incorporation in 60-d-old CD-treated rats was measured only in surviving individuals and most individuals of this group died by that time, the ^{3}H-T incorporation rate in this group presented here is likely to be an overestimate.

Histomorphometric Measurements. Necrosis. The volume density of necrotic hepatocytes at various time points after CCl_4 administration observed in 35-, 45-, and 60-d-old rats with different pretreatments is presented in Figure 6*a*, *b*, and *c*, respectively. Consistent with our serum enzyme data, no significant necrosis of hepatocytes was observed in the livers of rats injected with corn oil at any time point studied regardless of dietary treatment and age. Administration of CCl_4 did not result in a significant increase in volume density of necrotic hepatocytes at 6 h in 35- and 45-d-old ND-fed or CD-treated rats. Volume density of necrotic hepatocytes in 60-d-old rats was slightly increased by 6 h, but the increase was not significantly different between ND-fed and CD-treated rats (3.6 and 2.6%, respectively). Necrosis of hepatocytes induced by CCl_4 was slightly increased in ND rats of all the three age groups by 24 h and remained low or returned to the level before CCl_4 challenge by 48 h. In 45- and 60-d-old CD-treated rats, CCl_4-induced hepatic necrosis was greatly increased by 24 h and the necrotic injury was further increased by 48 h. In 35-d-old CD-treated rats, although CCl_4-induced hepatic necrosis was significantly increased by 24 h, there was no further increase at 48 h.

Mitosis. The hepatic mitotic activity, as estimated by volume density of hepatocytes in metaphase (shown in Fig. 7*a*, *b*, and *c* for 35-, 45-, and 60-d-old rats, respectively) in corn oil–injected groups remained fairly stable regardless of the dietary treatment and age. It should be noted that hepatic mitotic activity in 35-d-old rats was generally higher, although not statistically significant at all the time points studied, in comparison with 45- and 60-d-old rats regardless of the dietary treatment. There was a significant increase in hepatic mitotic activity in 45- and 60-d-old ND-fed rats 24 h after CCl_4 challenge (Fig. 7*b* and *c*). The adminis-

Table 2. *Hepatic disposition of CD in newborn and weaning rats after dietary treatment (30 ppm) of their dams*

CD concentration in dam's diet (ppm)	Starting day of diet after successful mating	Age of newborn rats (d)	Hepatic concentration of CD (μg/g liver)
30	7	2	48.9 ± 3.8
30	10	5	64.3 ± 1.4
30	5 postparturition	20	66.9 ± 12.9

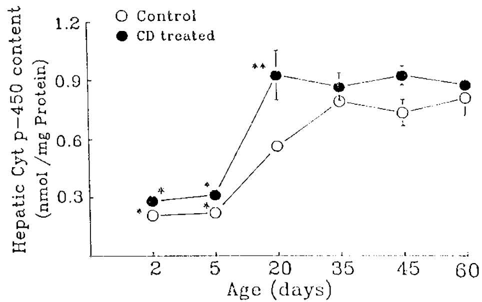

Fig. 1. Hepatic microsomal cytochrome P-450 (*Cyt p-450*) content in rats at various ages with or without dietary CD pretreatment. Each *point* represents the mean ± SEM of three to four measurements. Two or three livers were pooled for a single measurement in 2- or 5-d-old rats. *One asterisk* indicates a significant difference from 20-d-old and older rats with the same dietary treatment. *Two asterisks* indicate a significant difference between CD- and ND-fed rats of 20-d age group.

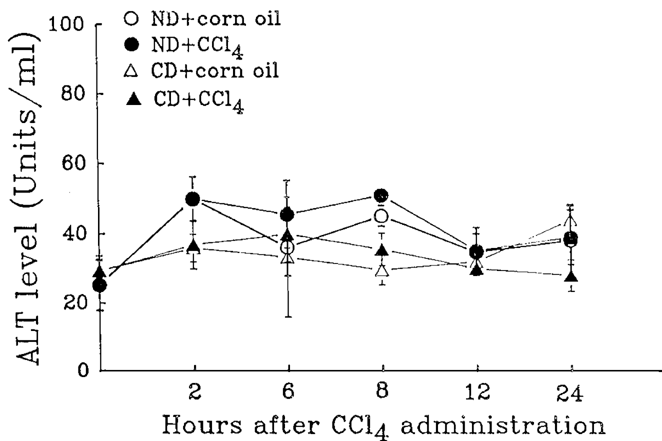

Fig. 2. Serum ALT levels over 24 h after the injection of CCl_4 (100 μL/kg) or corn oil in 2-d-old rats exposed to an ND or CD (30 ppm) diet through their dams for 15 d. The results represent the means ± SEM of at least four measurements. Serum from two or three pups was pooled for a single measurement.

tration of CCl_4 also induced an increase in mitotic activity in 35-d-old ND-fed rats, but it was not statistically significant due to the relatively high baseline level of mitotic activity in this age group. The difference in hepatic necrosis induced by CCl_4 at 24 and 48 h between 35-, 45-, and 60-d-old CD-treated rats (Fig. 6*a–c*) was commensurate with the difference in mitotic activity between these age groups. At 24 h, there was a significant increase in hepatic mitotic activity in 35-d-old CD-treated rats (Fig. 7*a*), whereas hepatic mitotic activity in 45- or 60-d-old CD-treated rats was not stimulated during the 48 h after CCl_4 administration (Fig. 7*b* and *c*).

In Vivo Metabolism of $^{14}CCl_4$. The data on the *in vivo* metabolism of $^{14}CCl_4$ are summarized in Table 3. Regardless of the age and dietary treatments, 80 to 85% of the administered $^{14}CCl_4$ was expired as unmetabolized parent compound within 6 h. CD pretreatment resulted in a significant increase in the proportion of administered $^{14}CCl_4$ expired as $^{14}CO_2$ compared with the ND-fed rats in the respective age groups, indicating an increased bioactivation of CCl_4 in CD-treated rats. Bioactivation of $^{14}CCl_4$ in 35-d-old CD-treated rats was significantly higher than in 45- or 60-d-old rats as indicated by a greater portion of the administered $^{14}CCl_4$ expired as $^{14}CO_2$. The total hepatic radioactivity and the radioactivity bound to tissue in 35-d-old CD-treated rats were significantly lower than in 60-d-old CD-treated rats but not significantly lower than in 45-d-old CD-treated rats.

DISCUSSION

Hepatotoxicity of CCl_4 in different age groups has been investigated in several studies. Because CCl_4 toxicity is dependent on prior hepatic activation, it was hypothesized in several studies that the newborn animals would be less sensitive to CCl_4-induced hepatotoxicity (39, 40). Although 1-d-old rats were found to be resistant to CCl_4-induced hepatic damage (39), rats as young as 4 d of age had extensive hepatic damage after CCl_4 treatment, and hepatic necrosis in 10-, 14-, and 21-d-old rats was of the same order of magnitude as observed in adults after CCl_4 treatment (40). A relatively high dose of CCl_4 (1 or 2 mL/kg) was used in the above-mentioned studies. It has also been reported that aging had no effects on hepatotoxicity of CCl_4 (41). The decreased metabolic activation of CCl_4 in aged rats (5-mo-old *versus* 20-mo-old rats) is counterbalanced by a diminished defense mechanism against peroxidative attack and the net result is no change in the extent of liver damage (41). In the present study, we found that newborn and developing rats are more resilient to hepatotoxicity of a low dose of CCl_4 as well as to its potentiation by pretreatment with CD.

Several possible mechanisms may contribute to the observed resilience to amplification of CCl_4 hepatotoxicity by CD in postnatally developing rats. Because hepatic deposition of CD in 2- and 20-d-old rats after 15 d of dietary exposure is comparable to that of adult CD-pretreated rats (Table 2), insufficient accumulation of CD in hepatic tissue could be excluded from the list of possible mechanisms for the lack of CD-potentiated CCl_4 toxicity. An important reason for the resilience to potentiation of CCl_4 hepatotoxicity and lethality in newborn rats could be less metabolic bioactivation of CCl_4. The level of hepatic microsomal cytochrome P-450, which is required for the initiation of CCl_4-induced liver damage, was much lower in 2-d-old CD-treated rats than in older CD-treated rats (Fig. 1), and this might explain the absence of CD-potentiated CCl_4 toxicity in that group. However, the cytochrome P-450 levels in 35-, 45-, and 60-d-old CD-treated rats were not significantly different (Fig. 1). Some isoenzymes of cytochrome P-450 have been reported to be more effective than others in metabolizing CCl_4 (42, 43), and they may develop differently with age. To examine such a possibility, which may be responsible for the observed differences in resilience to amplification of CCl_4 toxicity by CD among different age groups, *in vivo* metabolism of CCl_4 was investigated. Data on the *in vivo* metabolism of CCl_4 (Table 3) indicate that bioactivation of CCl_4 was not significantly different among the 35-, 45-, and 60-d-old CD-treated rats, whereas CCl_4 toxicity showed significant differences among these groups (Table 1 and Fig. 3). These results suggest that neither cytochrome P-450 level nor metabolic activation of CCl_4 could explain the absence of CD-potentiated CCl_4 hepatotoxicity and lethality in 35-d-old CD-treated rats.

It is apparent from the present study that the absence or presence of CD-potentiated CCl_4 hepatotoxicity and lethality is well correlated with the presence or absence of ongoing and stimulated hepatocellular regeneration and tissue repair. The serum enzyme and histomorphometric data (Figs. 3, 6, and 7) indicate that CCl_4 at a dose of 100 μL/kg did not result in significant liver damage in ND-fed rats regardless of age, whereas there was a stimulated or ongoing hepatic proliferative activity as early as 2 h in these groups as indicated by the ^{3}H-T incorporation data (Fig. 5). No hepatocellular regenerative or tissue repair activity was observed until 48 h in 60-d-old CD-rats (Fig. 5), in which remarkably potentiated CCl_4 hepatotoxicity and lethality were observed (Table 1 and Fig. 3). In contrast to the observation in 60-d-old CD-treated rats, CD treatment did not affect the ongoing hepatocellular proliferation in 35-d-old rats and a further increase of regenerative or tissue repair activity was noticeable at 24 h in that group, in which no lethality and much less toxicity of CCl_4 were observed. Previous studies (10, 14, 15)

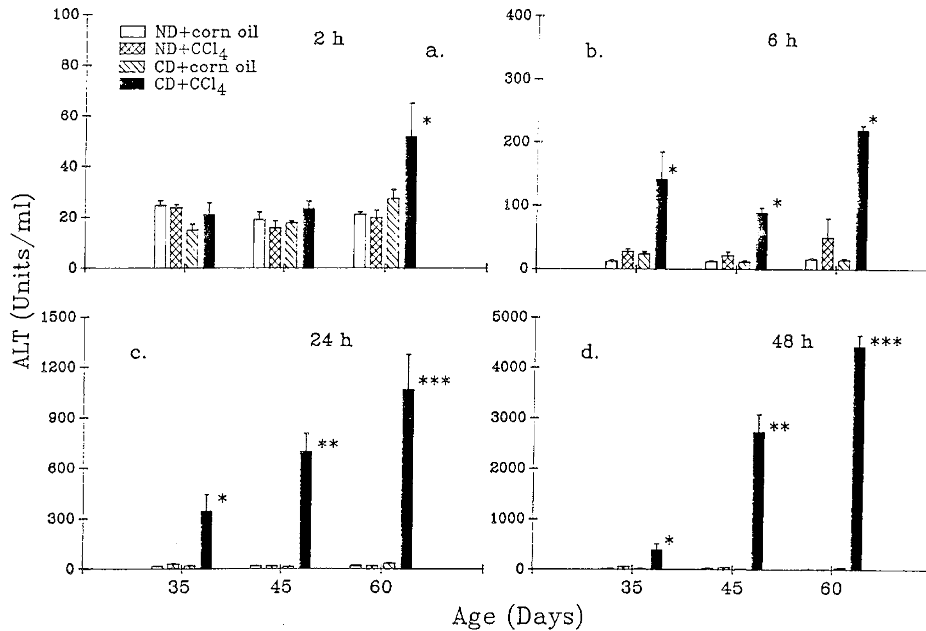

Fig. 3. Serum ALT levels at 2 (*a*), 6 (*b*), 24 (*c*), and 48 (*d*) h after the injection of CCl_4 (100 μL/kg) or corn oil in 35-, 45-, and 60-d-old rats fed either an ND or CD (10 ppm) diet. (Note the difference in scale for each of panels.) The results represent the means ± SEM of at least four rats. A single *asterisk* indicates a significant increase in ALT level as compared with corn oil–injected ND-fed rats at the same age. *Two asterisks* indicate that the ALT level is significantly higher than in 35-d-old CD+CCl_4 rats. *Three asterisks* indicate a significantly higher ALT level as compared with that in 35- or 45-d-old CD+CCl_4 rats.

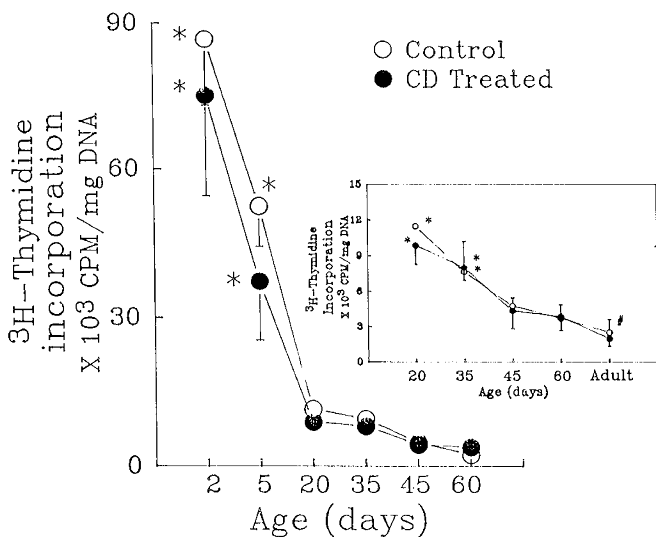

Fig. 4. ^{3}H-T incorporation in the hepatic nuclear DNA of rats of different ages fed either an ND or CD diet. ^{3}H-T was administered at a dose of 200 μCi/kg 2 h before the animals were killed. Each point represents the mean ± SEM of at least four rats. *One asterisk* indicates a significant difference from the rats older than 20 d with the same dietary treatment. The *insert* is a magnification of data for rats older than 20 d. The adult data are cited from reference 15 with permission from the authors.

have also shown that CD treatment does not alter ongoing or partial hepatectomy-stimulated hepatocellular regeneration.

It should be noted that liver injury measured by necrosis in 35- and 45-d-old rats is similar at 24 h in CD-treated rats (volume density of necrotic hepatocytes = 14.3 ± 1.3% and 18.3 ± 2.4%, respectively, Fig. 6*a* and *b*). This level of injury is prevented from progressing in 35-d-old rats as a result of simultaneously stimulated (3-fold) hepatocellular mitotic activity. In contrast, stimulation of mitotic activity in 45-d-old CD-treated rats was not evident (Fig. 7*b*). Consequently, despite a magnitude of liver injury similar to that observed in 35-d-old rats at 24 h (Fig. 6*a* and *b*), liver injury in 45-d-old rats becomes progressive at 48 h, whereas it is completely controlled in 35-d-old rats. Similarly, among CD-pretreated rats, a comparison of the magnitude of CCl_4-inflicted injury and the progression of that injury in 35-d-old and 60-d-old age groups *versus* the highly stimulated tissue repair in 35-d-old rats contrasted with absence of tissue repair in 60-d-old rats is illustrative of the decisive impact of hepatocellular regeneration and tissue repair mechanisms on the ultimate outcome of toxicity initiated by CCl_4.

These findings are supportive of the two-stage model of toxicity (44), wherein the early events responsible for initiating liver injury could be separated from subsequent biologic events that determine the final outcome of that injury. In the present study, a comparison of ND-fed and CD-treated rats in each age group indicates that the degree of the liver injury initiated by CCl_4 (serum enzyme data, Fig. 3) is proportional to metabolic activation and tissue binding of CCl_4 (Table 3). In each age group, CD treatment is associated with greater bioactivation of CCl_4. However, if one compares the liver injury initiated by CCl_4 and ultimate outcomes of the toxicity in various age groups, it is evident that the status of hepatocellular regeneration and tissue repair processes determine the progression or regression of the liver injury. In spite of very similar liver injury at 6 h when most CCl_4 has been exhaled and presumably the majority of bioactivation has taken place, the ultimate outcomes are dramatically different among 35-, 45-, and 60-d-old CD-treated rats. The most readily apparent feature among these age groups is the status of ongoing and stimulated tissue repair upon initiation of injury by CCl_4. The overall results suggest that the age-dependent CD potentiation of CCl_4 toxicity is related to the balance between the CCl_4-induced liver injury and the biologic responses such as hepatocellular proliferation and tissue repair leading to the recovery of liver injury rather than to the difference in the initial CCl_4-induced liver injury.

The mechanisms for the unsuppressed stimulation of cellular regeneration and tissue repair by CCl_4 in 35-d-old CD-treated rats are not understood at this time. Recent investigations reveal that some key factors such as TGF-α and TGF-β are involved in the regulation of regenerative activity after CCl_4-induced liver

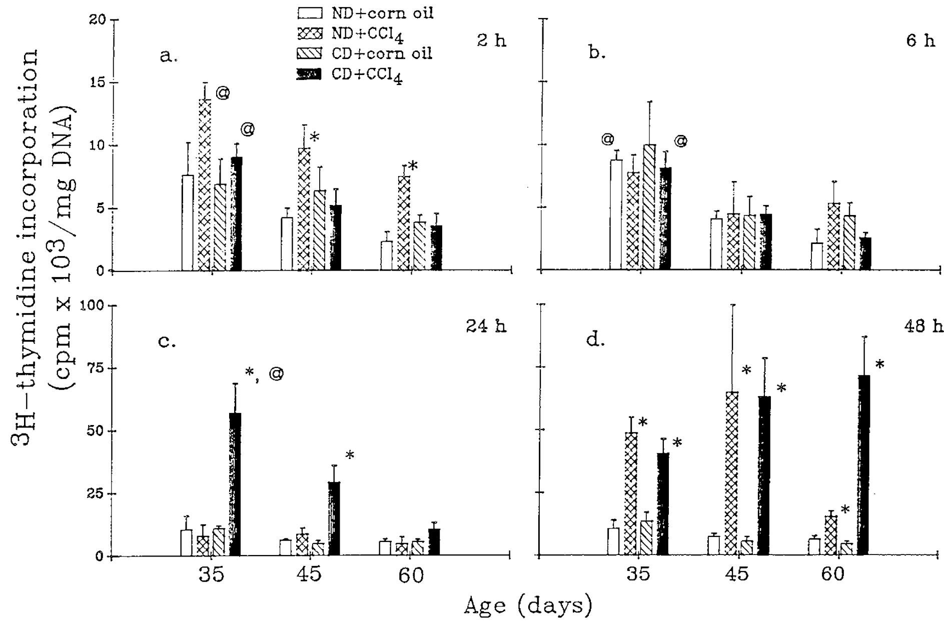

Fig. 5. ^{3}H-T incorporation in the hepatic nuclear DNA at 2 (*a*), 6 (*b*), 24 (*c*), and 48 (*d*) h after the injection of CCl_4 (100 μL/kg) or corn oil in 35-, 45-, and 60-d-old rats fed either an ND or CD diet. ^{3}H-T was administered at a dose of 200 μCi/kg 2 h before the animals were killed. The results represent the mean ± SEM of at least four rats. An *asterisk* indicates a significantly higher value as compared with the corn oil–injected rats with the same dietary treatment. @ indicates a significantly higher value compared with that in 45- or 60-d-old rats with the same treatment.

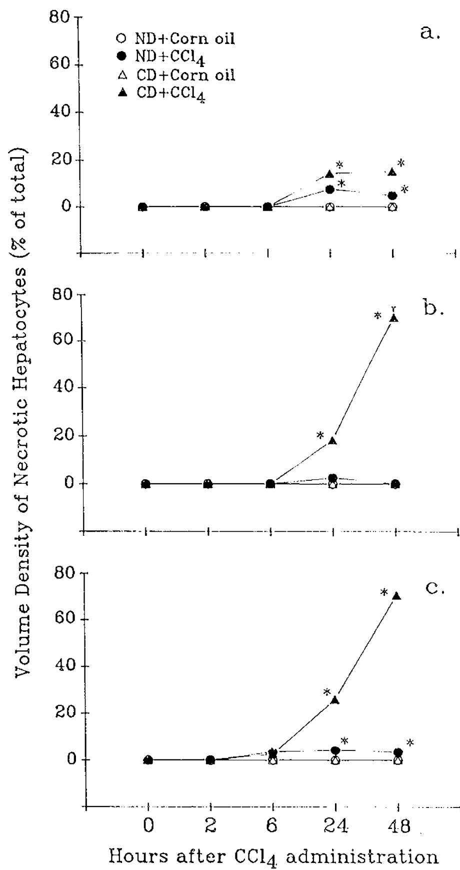

Fig. 6. Volume density of necrotic hepatocytes during a time course of 48 h after CCl_4 (100 μL/kg) or corn oil injection in 35-, 45-, and 60-d-old rats with or without CD dietary pretreatment (*a*, *b*, and *c*, respectively). Each *point* represents the mean ± SEM of 60 to 80 randomly selected areas. An *asterisk* indicates a significant difference from the corn oil–injected group with the same dietary treatment at the respective time point.

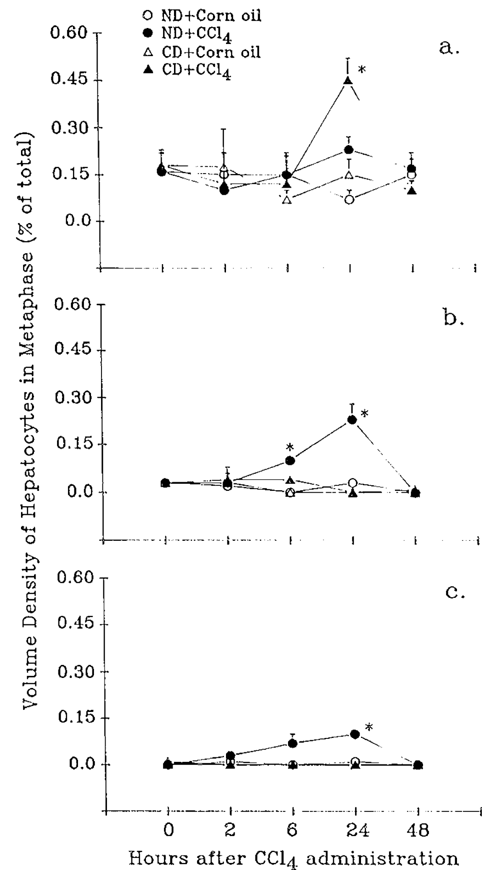

Fig. 7. Volume density of hepatocytes in metaphase during a time course of 48 h after CCl_4 (100 μL/kg) or corn oil injection in 35-, 45-, and 60-d-old rats with or without CD dietary pretreatment (*a*, *b*, and *c*, respectively). Each point represents the means ± SEM of 60 to 80 randomly selected areas. An *asterisk* indicates a significant difference from the corn oil-injected group with the same dietary treatment at the respective time point.

Table 3. *Metabolism of $^{14}CCl_4$ in rats at various ages**

Age (d)	Diet†	% of administered $^{14}CCl_4$ expired as free $^{14}CCl_4$‡	% of administered $^{14}CCl_4$ expired as $^{14}CO_2$	Hepatic radioactivity (dpm/g liver)	
				Total	Bound to tissue
35	ND	80.4 ± 7.2 (4)	0.31 ± 0.02	766 ± 50§	658 ± 38‖
35	CD	84.0 ± 2.1 (4)	0.53 ± 0.04¶**	1302 ± 190§	1067 ± 209‖
45	ND	83.9 ± 3.4 (3)	0.19 ± 0.01	1368 ± 562§	878 ± 256‖
45	CD	79.6 ± 1.4 (3)	0.30 ± 0.01¶	2078 ± 239	1888 ± 13
60	ND	84.7 ± 2.2 (3)	0.16 ± 0.03	2550 ± 189	2133 ± 174
60	CD	85.9 ± 4.5 (3)	0.36 ± 0.07¶	3516 ± 904	3179 ± 773

* Measurements of $^{14}CCl_4$ and $^{14}CO_2$ expiration were taken during 6 h at hourly intervals. The measurements of hepatic radioactivity were taken 6 h after the administration of $^{14}CCl_4$ (100 μL/kg) in corn oil vehicle.

† CD, 10 ppm CD diet.

‡ Numbers in parentheses are the total number of animals treated.

§ $p < 0.05$ compared with 60-d-old CD-fed rats in the same column.

‖ $p < 0.01$ compared with 60-d-old CD-fed rats in the same column.

¶ $p < 0.05$ compared with the ND group at the same age in the same column.

** $p < 0.01$ compared with all other groups in the same column.

injury (45, 46). One possible explanation for lack of suppressed cell division in younger rats is that expression of endogenous mitogen TGF-α is more easily stimulated as a result of toxicity induced by CCl_4. Another reason might be that expression of TGF-β, which regulates the level of mitotic activity stimulated by TGF-α, might be under tighter control during early postnatal development. Whether the age-dependent resilience to CD potentiation of CCl_4 hepatotoxicity is associated with differences in these factors among the different age groups is a question worth further investigation.

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Announcement

In view of the appalling events that have occurred in Somalia in the past several months, there is a recognized need in Somalia for medical expertise at the most basic level of pediatric and nutritional care. Moreover, this need is likely to exist for months and even years beyond resolution of the political and social events presently taking place.

Accordingly, you are being asked to join in a volunteer effort to provide medical care to an entire generation of infants and children in Somalia. Due to the current civil unrest in that country, there is no intent to begin provision of such care until order is restored. However, to avoid undue delay at that time, we are asking now for the names of volunteers. By organizing now, it will be possible to mount a meaningful effort within days that might otherwise require months. Because there are thousands of deaths weekly in Somalia, by starting now we may be able to save many thousands of children who will undoubtedly die without us.

Because it is likely that the need for our services will extend beyond a 12-month period, volunteers for 1 to 4 months (or longer), as schedules permit, within the next 24-month period will be gratefully accepted.

For more information, please contact: Karl S. Roth, M.D., or Festus O. Adebonojo, M.D., Department of Pediatrics, Medical College of Virginia, Childrens Medical Center, Box 239, Richmond, VA 23298-0239.