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Liver Nuclear DNA Synthesis in Mice Following Carbon Tetrachloride Administration or Partial Hepatectomy[1]

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Abstract. Long-term, continuous (twice per week) administration of CCl_4 to male mice resulted in a high incidence of liver nodules which appeared to be resistant to the necrotizing effects of CCl_4 but showed no features of malignant neoplasia. Liver nuclear DNA synthesis was compared in mice given CCl_4 and in mice subjected to partial hepatectomy (PH). Mice were given by gavage corn oil or CCl_4 in corn oil for periods of 2 to 25 weeks and several mice were subjected to PH after 12 and 25 weeks of corn oil treatment. Mice were given [^{3}H]TdR during liver regeneration and newly synthesized liver nuclear DNA was isolated and separated by BND–cellulose chromatography. Greater than 85% of the labeled DNA from PH mice eluted from BND–cellulose columns as double-stranded (ds) DNA with single-stranded (ss) regions or ends and less than 15% as ds DNA. When mice were treated with CCl_4 for 8 weeks or longer a significantly greater portion of liver nuclear DNA eluted as ds DNA. Administration of HU and 5-FU with [^{3}H]TdR decreased [^{3}H]TdR incorporation into DNA to low levels incompatible with unscheduled DNA synthesis. Single doses of CCl_4 given to mice treated with corn oil for 2 to 12 weeks provided newly synthesized DNA which was primarily (>80%) ds DNA with ss regions or ends, but after 25 weeks of corn oil administration, a single dose of CCl_4 resulted in newly synthesized DNA with a greater proportion of ds DNA. The high labeling of ds DNA in mice treated with CCl_4 may have resulted from an alternate pathway of DNA synthesis catalyzed by the enzymes or enzyme complexes associated with semiconservative DNA synthesis or from proliferation of nonparenchymal cells with a rapid turn-over rate.

Long-term carbon tetrachloride (CCl_4) administration to mice and to rats results in a high incidence of liver nodules which have been described as varying from hyperplastic nodules to hepatocellular carcinoma (1). CCl_4 also promotes liver tumor formation, as does partial hepatectomy (PH), when given after an initiating dose or regimen of a liver carcinogen (2–4). It is not known whether liver DNA synthesis is modified by single doses of CCl_4 or by long-term CCl_4 administration.

During a series of experiments involving the adaptation of the liver to long-term continuous CCl_4 administration to mice, a change was observed in liver nuclear DNA synthesis which suggested the utilization by regenerating liver cells of an alternate pathway of DNA synthesis in addition to semiconservative DNA synthesis. This alternate pathway appeared to differ from non-semiconservative (repair or unscheduled) DNA synthesis which follows alkylation or radiation damage to DNA. A similar change in liver nuclear DNA synthesis also occurred in older mice when liver regeneration was induced by a single dose of CCl_4. Regeneration induced by PH, even in older mice, was not accompanied by any deviation from the expected pattern of liver nuclear DNA synthesis. The results of these experiments are presented and discussed in this report.

Materials and Methods. Male CD-1 mice weighing 20–25 g, obtained from Charles River Canada, St. Constant, Quebec, were housed in the research animal facilities and given constant access to water and to food (Purina Lab Chow, Ralston–Purina Co., St. Louis, Mo.). A reverse lighting schedule was maintained so that the dark period occurred from 6:30 AM to 6:30 PM and the light period from 6:30 PM to 6:30 AM. Sucrose, technical grade CCl_4, and reagent grade NaI were obtained from Fisher Scientific Company, Pronase from Calbiochem, benzoylated naphthoylated DEAE–cellulose (Servacil BND)

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from Accurate Chemical and Scientific Company, Hicksville, New York. Tris–HCl, ethidium bromide, 5-fluorouracil (5-FU), hydroxyurea (HU), and S_1 nuclease were from Sigma Chemical Company, St. Louis, Missouri. [*Methyl*-^{3}H]Thymidine ([^{3}H]TdR) was obtained from Amersham Radiochemicals, Inc., Chicago, Illinois or from New England Nuclear Corporation, Boston, Massachusetts.

Administration of CCl_4. Mice were given CCl_4 chronically in the following schedule: A CCl_4/corn oil (Mazola) mixture was given orally twice a week on a Monday–Thursday sequence. The doses given were 0.05 μl (0.335 μmole) CCl_4/g body wt in the first week, 0.10 μl (0.67 μmole) CCl_4/g body wt in the second week, 0.15 μl (1.005 μmole) CCl_4/g body wt in the third week, and from the fourth week through the remainder of the experimental period 0.3 μl (2.01 μmole) CCl_4/g body wt.

Mice in another large group were given 0.1 ml of corn oil in the same sequence throughout the experimental period.

Single doses of the CCl_4/corn oil mixture were given to mice which had been treated with corn oil for 2, 4, 8, 12, and 25 weeks. Mice were given the CCl_4/corn oil mixture by gavage to provide 0.07 μl (.469 μmole) CCl_4/g body wt.

CCl_4 administration was timed so that [^{3}H]TdR injections could be given at intervals of 18, 42, and 72 hr after the chlorinated hydrocarbon had been given and always 2 1/2–3 hr into the dark cycle.

Partial hepatectomy (PH). Partial hepatectomies were performed on mice which had been treated with corn oil for 12 or 25 weeks. Under ether anesthesia, 60–70% of the liver mass was removed. Surgery always was performed in the afternoon so that injections of [^{3}H]TdR could be given approximately 42 hr later, 2 1/2 hr into the dark cycle. We have found, as reported by Chernozemski and Warwick (5), that maximum rates of liver DNA synthesis in mice occur between 36 and 42 hr after PH.

Administration of [^{3}H]TdR. The protocol described by Bowden *et al.* (6) was adapted for labeling liver nuclear DNA. [^{3}H]TdR was mixed with 0.154 *M* NaCl solution to make a solution containing 30 μCi [^{3}H]TdR/0.1 ml. [^{3}H]TdR administration was begun 42 hr after PH, after a single dose of CCl_4 or after the last dose in a series of continuous CCl_4 administration. Several CCl_4-treated mice also were given [^{3}H]TdR 18 and 72 hr after a single dose or the last dose of CCl_4 in a continuous schedule. Mice were given six intraperitoneal injections of 0.1 ml of the [^{3}H]TdR solution at 1/2-hr intervals. When HU and 5-FU were included, mice were first given an intraperitoneal injection of 2.5 mg HU and 0.5 mg 5-FU in 0.1 ml 0.154 *M* NaCl; one-half hr later the series of intraperitoneal doses was begun, each dose at 1/2-hr intervals and each dose providing 2.5 mg HU, 0.5 mg 5-FU, and 30 μCi [^{3}H]TdR. Mice were killed 1/2 hr after the last injection by decapitation and exsanguination and the liver was removed and prepared for extraction of liver nuclear DNA.

DNA extraction and determination of radioactivity. The liver from each mouse was rinsed in iced buffer (0.25 *M* sucrose, 10 m*M* Tris–HCl, pH 7.9, 3 m*M* $CaCl_2$, 0.1% Triton X-100) and then homogenized in another 20-ml aliquot of the buffer. Liver nodules were removed and were not included in the homogenate. A nuclear precipitate was obtained by centrifugation at 2000 rpm in a Sorvall RC-2B refrigerated centrifuge with the SS-34 rotor. Nuclear DNA was isolated using the method of Endow *et al.* (7) excluding the final purification by ultracentrifugation.

DNA specific radioactivity (SRA) was determined either by (i) ethidium bromide fluorescence followed by precipitation of DNA on GF/C filters for liquid scintillation spectrometry (8) or (ii) by hydrolyzing a portion of the DNA extract with 5% trichloroacetic acid (TCA), determining DNA concentration with the method of Burton (9) and radioactivity by adding an aliquot of the hydrolysate to Aquasol (New England Nuclear Corp., Boston, Mass.) for liquid scintillation spectrometry. Counting efficiency varied from 20 to 27%.

DNA damage. The method of Cox *et al.* (10) as modified by Laishes *et al.* (11) was used to determine liver DNA damage. Liver DNA was labeled by administering [^{3}H]TdR to neonatal mice (11) and DNA damage was determined when the mice were 4 months of age. CCl_4 in doses ranging from 0.02 to 0.10 μl (0.134 to 0.67 μmole) CCl_4/g body wt was administered by gavage and controls were given corn oil by gavage. Mice were killed at

intervals of 4 to 72 hr after treatment, the livers were gently macerated in hypotonic buffer and a small quantity was placed on a linear alkaline sucrose gradient for centrifugation as described by Cox *et al.* (10). The lysing solution at the top of the gradient contained sodium sarcosinate (Sarkosyl) in place of SDS but other procedures were identical to those of Cox *et al.* (10).

BND–cellulose chromatography. BND–cellulose chromatography was performed with the following modifications of the method of Hensen (12). BND–cellulose was washed once in 20% methanol, then five times with 2 *M* NET buffer (NET buffer is X *M* NaCl, 1 m*M* EDTA, 10 m*M* Tris–HCl (pH 7.4)) and was stored in 0.3 *M* NET buffer at 4°C. Columns approximately 1 cm in diameter and 2.5 cm in height were formed in 2.5-ml disposable plastic syringes and the BND-cellulose columns were washed extensively with the 0.3 *M* NET buffer.

All samples of DNA which were subjected to BND–cellulose chromatography were taken from mice injected with [^{3}H]TdR 42 hr after PH, after a single dose of CCl_4 or after the last dose in a continuous schedule. Each sample of liver nuclear DNA, dissolved in 0.3 *M* NET buffer, was divided into three portions and DNA was precipitated with the addition of iced methanol. DNA in two of the portions was redissolved in pH 4.5 buffer (0.1 *M* NaCl, 30 m*M* sodium acetate, 0.01 m*M* $ZnSO_4$, pH 4.5) and the third in 0.3 *M* NET buffer. S_1 nuclease was added to one of the samples dissolved in pH 4.5 buffer and all three samples were incubated for 60 min at 37°C. DNA was precipitated with iced ethanol and dissolved in 0.3 *M* NET buffer.

An aliquot of the solution containing 30 to 50 μg DNA was passed five times through a 20-gauge hypodermic needle to shear the DNA and the DNA was then adsorbed on a BND–cellulose column. The column was eluted sequentially with 10 ml of each of the following buffers: fraction 1—0.3 *M* NET buffer; fraction 2—1.0 *M* NET buffer; and fraction 3—1.0 *M* NET buffer + 1.8% caffeine. TCA-precipitatible material from each fraction was collected on a GF/C filter and radioactivity was determined by liquid scintillation spectrometry. DNA eluting in fraction 2, 1.0 *M* NET buffer, was considered double-stranded (ds DNA) without single-stranded regions or ends. DNA eluting from fraction 3, 1.0 *M* NET buffer + 1.8% caffeine, is ds DNA with single stranded regions or ends (ds DNA with ss regions).

Statistical comparisons were made using a computerized program for the analysis of variance.

Results. *Liver nodule incidence and histological features.* Liver nodules were found in 8 of 12 mice treated continuously with CCl_4 for 25 weeks. These nodules varied in size from small punctate lesions to those which weighed 0.8 g; in 7 of the 8 mouse livers there were multiple nodules. Histologically the nodules were composed of cords of hepatocytes without centrilobular veins or portal triads.

There was none of the hepatocyte necrosis and inflammation which was so prominent in the surrounding non-nodular liver tissue, the hepatocytes showed no atypical features, and there was no evidence of invasion or metastasis. The nodules were considered adenomas or Type I according to the Becker classification of mouse liver nodules or tumors (13).

Specific radioactivity (SRA) of DNA and effects of HU. The SRAs of DNA from the control and various treatment groups are summarized in Table I. The data conform to the results previously reported by Smuckler and Gans (8) although the DNA SRAs in this current series of experiments were somewhat higher. The combination of HU plus the nucleotide analog 5-FU reduced DNA SRA to low levels.

DNA damage. Liver nuclear DNA damage could be demonstrated following a single dose of CCl_4 (Fig. 1). A shift to DNA of lower density was greatest 18 hr after the administration of CCl_4 and a normal pattern of sedimentation was restored within 24 to 36 hr. These changes were observed only following doses of CCl_4 which resulted in liver necrosis. Doses of CCl_4 which did not produce necrosis did not result in a shift in the sedimentation of DNA.

BND–cellulose chromatography. [^{3}H]TdR-labeled liver nuclear DNA from PH mice eluted primarily (>85%) as ds DNA with ss regions and less than 15% as ds DNA (Fig. 2). When mice were given single doses of CCl_4 after 2, 4, 8, or 12 weeks of corn oil administration, liver nuclear [^{3}H]DNA eluted from

TABLE I. EFFECTS OF HYDROXYUREA (HU) ON THE SPECIFIC RADIOACTIVITY (SRA) OF LIVER NUCLEAR DNA

Treatment	HU + 5-FU	SRA (cpm/μg DNA) 19 hr[a]	42 hr[a]	72 hr[a]
Control	−		40 ± 7 (6)	
	+		<1 (6)	
PH	−		473 ± 55 (6)	
	+		14 ± 6 (6)	
Acute CCl_4	−	14.5 ± 3.8 (4)	531 ± 56 (9)	356 ± 54 (5)
	+	1.8 ± 0.7 (4)	31 ± 7 (9)	32 ± 12 (5)
Chronic CCl_4	−	50, 22	457 ± 42 (8)	394, 430
	+	6.8, 6.2	55 ± 11 (8)	25, 24

Note. The results in this and the next table are expressed as the mean ± 1 standard error of the mean. Numbers in parentheses show the numbers of mice in each group. Significance of the differences between the means: PH with HU + 5-FU vs acute CCl_4 with HU + 5-FU, $P < 0.10$; PH with HU + 5-FU vs chronic CCl_4 with HU + 5-FU, $P < 0.01$; acute CCl_4 with HU + 5-FU vs chronic CCl_4 with HU + 5-FU, $P < 0.05$.

[a] Time in hours when [^{3}H]TdR was administered following PH, after a single dose of CCl_4 or after the last dose of corn oil or CCl_4.

BND–cellulose columns in the same proportions as DNA from PH mice (Fig. 2).

Changes in the elution of DNA from the BND–cellulose columns were observed in liver nuclear [^{3}H]DNA from mice treated chronically with CCl_4 for 8 weeks or longer. After 12 weeks of treatment with CCl_4 there was a significantly greater percentage of ds DNA than from mice treated acutely with CCl_4 or from PH mice (Fig. 2). Liver nuclear [^{3}H]DNA from mice treated chronically with CCl_4 for 25 weeks also eluted with a significantly greater percentage as ds DNA than did liver nuclear [^{3}H]DNA from mice subjected to PH (Fig. 2). When mice treated chronically with CCl_4 for 25 weeks were given HU plus 5-FU with [^{3}H]TdR, liver nuclear [^{3}H]DNA eluted from the BND–cellulose columns in a manner similar to liver nuclear [^{3}H]DNA from mice treated with CCl_4 but not given HU and 5-FU in combination with [^{3}H]TdR (Fig. 2). Of the liver nuclear [^{3}H]DNA from mice given corn oil for 25 weeks and then treated acutely with CCl_4, significantly more DNA eluted as ds DNA than did liver nuclear [^{3}H]DNA from mice given corn oil for 25 weeks and then subjected to PH (Fig. 2).

Greater than 85% of the liver nuclear [^{3}H]DNA incubated with S_1 nuclease eluted as ds DNA regardless of the treatment regimen (Table II). These results show that DNA in fraction 3 was ds DNA containing ss regions.

Discussion. BND–cellulose chromatogra-

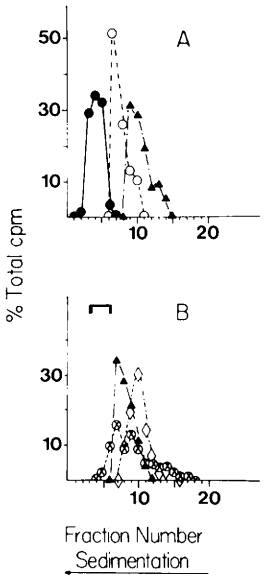

FIG. 1. Alkaline sucrose gradient profiles of liver DNA. (A) Effect of CCl_4 dose on liver DNA sedimentation. Control liver: ●; mice treated with CCl_4: ○, 0.05 μl CCl_4/g body wt and ▲, 0.1 μl CCl_4/g body wt. All mice were killed 18 hr after being given corn oil or CCl_4. (B) Time course of changes in DNA sedimentation. Mice given 0.1 μl CCl_4/g body wt and killed: ⊗, 4 hr; ▲, 6 hr; and ◇, 18 hr. Liver DNA from mice killed 24 hr after CCl_4 had sedimentation profiles which either could be superimposed on that for 6 hr and more often on that of controls and, therefore, are not shown. The sedimentation of [^{3}H]DNA always was similar to controls 36 hr after CCl_4. The bracket indicates the limits of sedimentation of liver DNA from nine control mice.

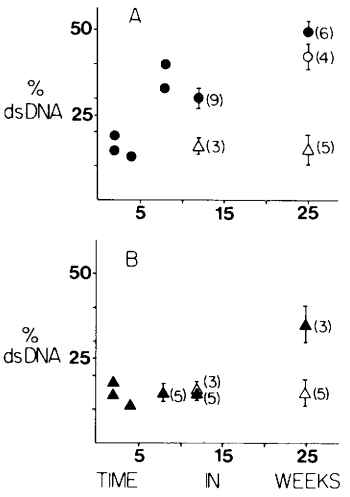

FIG. 2. Percentage of newly synthesized DNA eluted as ds DNA from BND–cellulose column. (A) Δ, Partial hepatectomy (PH); ●, continuous CCl_4 administration; O, continuous CCl_4 administration, given HU and 5-FU with [^{3}H]TdR. The bars indicate ±1 standard error of the mean when N = at least 3; numbers in parentheses are the numbers of mice at each point. (B) Δ, Partial hepatectomy (same mice as in A); ▲, single dose of CCl_4. Significance of the differences between the means: 12 weeks: PH vs acute CCl_4, $P > 0.10$; PH vs chronic CCl_4, $P < 0.005$; acute CCl_4 vs chronic CCl_4 $P < 0.001$. For 25 weeks: PH vs acute CCl_4, $P < 0.01$; PH vs chronic CCl_4, $P < 0.001$; acute CCl_4 vs chronic CCl_4, $P > 0.05$.

phy has been used as an effective method for determining repair or unscheduled DNA synthesis (14). Newly synthesized DNA in replicating cells is assembled initially into small units as short ss strands and are recovered as short strands of ds DNA with ss regions or ends (15–17). The newly synthesized units, reflecting semiconservative DNA synthesis, would be recovered primarily in 1.0 M NET buffer + 1.8% caffeine (fraction 3 in the elution sequence used in these experiments) and this fraction would contain the greater (>80%) amount of [^{3}H]DNA. Any significant labeling of ds DNA in this system would be compatible with the repair of DNA lesions which had been produced, for example, either by alkylation or radiation damage to DNA. Repair or unscheduled DNA synthesis should represent a significant component of total DNA synthesis and should not be inhibited by the administration of hydroxyurea (14, 18).

Craddock and Henderson (19) reported that DNA damage and repair, which they observed in liver following the administration of CCl_4 to rats, was not the same as alkylation damage to DNA with subsequent repair by unscheduled DNA synthesis. They suggested that CCl_4-induced disruption of cytoplasmic organelles liberated DNAases as well as other tissue destructive hydrolases (19). The data on CCl_4-induced DNA damage in mouse livers (Fig. 1) are compatible with the idea that DNA strand breaks occurred as a part of the generalized tissue destruction, not from specific alkylation damage to DNA. It is not clear whether DNAase-induced strand breaks occurred in cells which survived and by what process these breaks may have been repaired. Other studies have shown that CCl_4 did not induce unscheduled DNA synthesis in rat hepatocytes in primary culture (20, 21).

Liver regeneration during continuous CCl_4 administration, in contrast to regeneration following PH, produced newly synthesized DNA with a high proportion of ds DNA. The low DNA SRA following the administration of HU and 5-FU to mice treated continuously with CCl_4 indicated that unscheduled DNA synthesis did not account for the increased [^{3}H]TdR incorporation into ds DNA. Moreover, [^{3}H]TdR was given, in the experiments reported here, at a time period in which no damaged DNA should be present (Fig. 1; (22)). The processes which resulted in the high proportion of ds DNA are not known. Repetitive cycles of necrosis and regeneration during long-term CCl_4 administration may have fostered adaptative changes in the DNA repli-

TABLE II. EFFECT OF S_1 NUCLEASE DIGESTION ON BND–CELLULOSE CHROMATOGRAPHY OF LIVER NUCLEAR [^{3}H]DNA

Group	S_1 Nuclease	Percentage added [^{3}H]DNA eluted into fraction[a]		
		1	2	3
PH	–	0	10.3	89.7
	+	0	88.4	11.6
Acute CCl_4 (12 weeks)	–	0	14.9	85.1
	+	0	89.3	10.7
Chronic CCl_4 (25 weeks)	–	0	48.6	51.4
	+	0	92.6	3.8

[a] Fraction 1—0.3 M NET buffer; fraction 2—1.0 M NET buffer; fraction 3—1.0 M NET buffer + 1.8% caffeine.

cating enzyme complexes thereby providing an alternate pathway of semiconservative DNA synthesis.

The heterogeneity of cell populations in the livers of mice treated chronically with CCl_4 (8) adds further uncertainties. Nonparenchymal cells (NPCs) constitute 50% of the cells in which DNA synthesis occurs 42 hr after the last dose of CCl_4 (8). If NPCs have a much more rapid turnover than hepatocytes, the schedule of [^{3}H]TdR administration would have allowed a considerable number of NPCs to complete DNA replication by semiconservative DNA synthesis. NPC DNA would have contributed much of the ds DNA in the livers of mice given long-term treatment with CCl_4.

Time was another important component in the change in DNA synthesis. That the liver adapts to repetitive injury by CCl_4 is evident (i) in the new pattern of liver nuclear DNA synthesis which appears during the course of repetitive CCl_4 administration, possibly as a result of the proliferation of NPCs and (ii) in the emergence of nodules of liver cells which appear to be resistant to the necrotizing effects of CCl_4. Some of the adaptative changes induced by continuous CCl_4 administration apparently occur spontaneously as mice grow older, since regeneration following a single dose of CCl_4 to older mice included a high level of [^{3}H]TdR incorporation into ds DNA.

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